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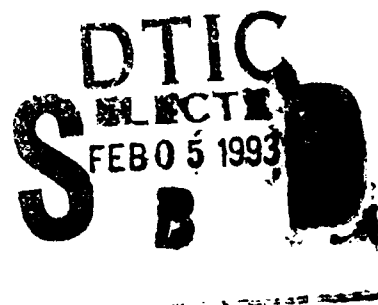


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Laboratory Note No. 82

**PYRIDOXINE DEFICIENCY AND WOUND HEALING IN RATS:
ASSESSMENT OF ANTIOXIDANT ENZYMES AND
IMMUNOLOGICAL STATUS**

Thornton Samuel Mu
and
Michael A. Dubick



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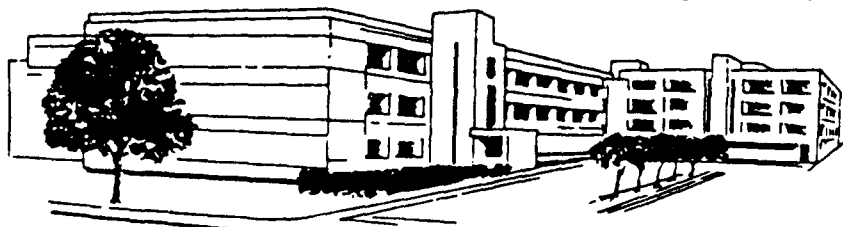


Division of Military Trauma Research

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Pyridoxine Deficiency and Wound Healing in Rats: Assessment of Antioxidant Enzymes and Immunological Status -- Thornton S. Mu and Michael A. Dubick

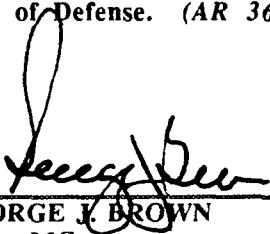
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GEORGE J. BROWN
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Many nutrients are involved in the complex process of wound healing. Of the vitamins, pyridoxine (vitamin B₆) deficiency has resulted in impaired healing, primarily due to its effect on amino acid and protein metabolism. This study examined the effects of pyridoxine deficiency on wound healing and its effects on acute phase proteins, immunoglobulins, and antioxidant enzymes in rats. Female rats were fed diets containing 0 mg/kg (deficient), 0.25 or 1.0 mg/kg (marginally deficient) or 7 mg/kg (control) pyridoxine for 5 weeks. Half the animals in each dietary group received a 3 cm long full skin incisional wound on the back and all animals were euthanized 1 week later. The results showed that despite pyridoxine deficiency, the animals in each dietary group had similar acute phase responses and antioxidant enzyme status in response to the injury.					
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ABSTRACT

Many nutrients are involved in the complex process of wound healing. Of the vitamins, pyridoxine (vitamin B₆) deficiency has resulted in impaired healing, primarily due to its effect on amino acid and protein metabolism. This study examined the effects of pyridoxine deficiency on wound healing and its effects on acute phase proteins, immunoglobulins, and antioxidant enzymes in rats. Female rats were fed diets containing 0 mg/kg (deficient), 0.25 or 1.0 mg/kg (marginally deficient) or 7 mg/kg (control) pyridoxine for 5 weeks. Half the animals in each dietary group received a 3 cm long full skin incisional wound on the back and all animals were euthanized 1 week later. The results showed that despite pyridoxine deficiency, the animals in each dietary group had similar acute phase responses and antioxidant enzyme status in response to the injury.

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FOREWORD

The work summarized in this report was part of an Inhouse Laboratory Independent Research project in the Division of Military Trauma Research. The work was performed by Mr. Mu during his tenure in the Division as part of the George Washington University-Department of Defense Science and Engineering Summer Apprenticeship Program for high school students.

**PYRIDOXINE DEFICIENCY AND WOUND HEALING IN RATS:
ASSESSMENT OF ANTIOXIDANT ENZYMES AND IMMUNOLOGICAL
STATUS--Thornton Samuel Mu and Michael A. Dubick, Ph.D.**

Wound healing is a complex process that takes place in many stages (1) and requires the presence of many important hormones, nutrients, and proteins, such as acute-phase proteins, immunoglobulins, and proteins and enzymes that regulate potentially damaging oxygen radicals (2-5). These oxygen radicals formed in response to trauma and tissue injury can cause serious cell injury, such as lipid peroxidation (6).

Among the nutrients, pyridoxine (vitamin B₆) plays an important role in amino acid and protein metabolism, including the synthesis and maturation of connective tissue proteins (7). Consequently, pyridoxine deficiency can affect normal wound healing (8). Pyridoxine also possesses an indirect antioxidant function that prevents damage done by oxygen radicals (9), functions as a coenzyme in the metabolism of amino acids (10), and is important for normal immune function (11). Because proteins, such as immunoglobulins, antioxidant enzymes, and acute-phase proteins, are composed of amino acids, it is assumed that a deficiency in vitamin B₆ would adversely affect production of these proteins.

An evaluation of combat casualties indicates that penetrating injuries account for up to 90% of all casualties (12). Although many of these are not life threatening, circumstances that impair the healing process can expend considerable military medical resources and delay the recovery of the injured soldier. Considering the potential importance of adequate pyridoxine nutriture to the healing process and the indication that about 50% of the U.S population consumes less than 70% of the RDA for vitamin B₆, a better understanding of the role of pyridoxine in wound healing could lead to improved therapeutic regimens or preventive measures, thus reducing morbidity and the consumption of medical resources by the injured patient.

We proposed, therefore, that pyridoxine deficiency would impair the ability to respond to injury. In this study we examined the effects of various stages of

pyridoxine deficiency on the status and presence (activity) of antioxidant enzymes and on the concentrations of certain acute-phase proteins and immunoglobulins in an incisional wound model in the rat.

MATERIALS and METHODS

Experimental protocol

Young adult, female Long-Evans rats, initially weighing 175-200 g were employed in these studies. A total of twenty-four rats were used in this phase of the study. The rats were randomly assigned to four groups of six rats. The first group was fed a diet devoid of (deficient) vitamin B₆. The second group was fed a diet with 0.25 mg vitamin B₆ per kg of diet. The third group was fed a diet with 1 mg/kg vitamin B₆. The fourth group was fed a control diet with 7 mg/kg vitamin B₆. All four groups were fed their respective diets for a period of five weeks. Three rats within each group underwent surgery in which an incision was made through the full thickness of the skin on the dorsal side. The rats recovered for one week after the surgery and were fed their respective diets during the recovery period.

Biochemical Studies

At the end of six weeks, the rats were overdosed with pentobarbital (120 mg/kg). Blood was withdrawn by cardiac puncture into citrated tubes and plasma was recovered by centrifugation for further evaluation of vitamin B₆ levels, immunoglobulin G and M, albumin, and fibrinogen. Liver, kidney, and brain were quickly removed, snap frozen in liquid nitrogen, and stored at -70°C in a biological freezer until assayed.

In preparation for assays to determine glutathione peroxidase, glutathione reductase, superoxide dismutase, and protein content, samples of kidney, liver, and brain were homogenized separately using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in a homogenizing buffer composed of 0.25M sucrose and 10mM Tris-HCl, pH 7.4 for ten seconds. Using a Branson sonifier cell disruptor (Branson Ultra Sonic, Danbury, CT), the samples were sonicated three times

for a period of five seconds each. The samples were centrifuged for 30 minutes at 10,000 g in a RC-5 Superspeed Refrigerated Centrifuge (DuPont Instruments, Newton, CT).

The samples were tested for glutathione peroxidase by using hydrogen peroxide as a substrate (13) and tested for glutathione reductase activity (14) by measuring the disappearance of beta-nicotinamide adenine dinucleotide phosphate (beta-NADPH). Total superoxide dismutase (SOD) (13) activity was measured by the ability of the enzyme to inhibit the auto-oxidation of pyrogallol. Manganese SOD activity (13) was measured under the same conditions except the buffer contained 1mM KCN. CuZnSOD was calculated by subtracting MnSOD activity from the total SOD activity. All spectrophotometric assays used the Beckman DU-70 Spectrophotometer (Beckman Instruments, Fullerton, CA).

Immunoglobulin G and M (IgG and IgM, respectively) concentrations in plasma were determined in diffusion plates (Kallestad, Chaska, MN) by double immunodiffusion (15). The results were recorded after 24 and 48 hrs incubation but only the 48 hr values were used according to standard protocol in clinical laboratories. Ten μ L of antibody was placed in the center well with dilutions of plasma placed in the outer wells. For IgM, neat through 1/32 dilutions were used and for IgG, 1/32 to 1/1024 dilutions were used.

Fibrinogen and albumin concentrations were determined by rocket immunoelectrophoresis (16) using the Bio-Rad horizontal electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Albumin concentrations were determined from standards, and fibrinogen concentrations were calculated as arbitrary units based on dilutions of normal rat plasma.

Protein content was determined with a commercially available kit (Bio-Rad, Richmond, CA) using the Beckman Du-70 spectrophotometer.

Statistical Analysis

Data were analyzed by two way ANOVA with injury and diet as factors. Significant differences were

further evaluated by the Newman-Keuls method for multiple comparisons. A $p < 0.05$ was considered statistically significant.

Animal Use Statement

Animal quarantine and prior care were performed in accordance with SOP# OP-ARG-4 and OP-ARG-40 and conforms to the provisions of the NIH as stated in the Guide for the Care and Use of Laboratory Animals (NIH publication #85-23).

RESULTS

Pyridoxine status

Tests to determine pyridoxine status in rats showed that rats with no vitamin B₆ in their diets were indeed deficient in vitamin B₆, whereas the control rats with 7mg/kg of pyridoxine had normal vitamin B₆ concentrations in their plasma. The other two groups had intermediate vitamin B₆ levels in their plasma (data not shown).

Acute-phase proteins (Table 1)

Concentrations of fibrinogen were not significantly affected by either diet or injury whereas albumin concentrations were significantly lower in rats with injury ($p = 0.0023$) and those fed pyridoxine deficient diets ($p = 0.0002$).

Immunoglobulin G and M (Table 2)

Concentrations of IgG and IgM in plasma were not significantly different among the dietary groups. Plasma immunoglobulin concentrations in injured rats were also not significantly different from those in noninjured rats within each dietary group.

Antioxidant enzyme status (Table 3)

Activity of glutathione peroxidase per gram of tissue (GPx/g) was significantly higher ($p = 0.0240$) in the livers of rats deficient in vitamin B₆ compared with controls. However, in brain and kidney samples,

GPx activity was not significantly affected by the different vitamin B₆ diets. In all dietary groups, GPx activity from brain samples from injured rats showed a significant decrease ($p=0.0229$) compared with their uninjured counterparts, whereas in their liver and kidney samples, a nonsignificant trend of decrease was noted.

In the rats that were deficient in vitamin B₆, activity of glutathione reductase per gram of tissue (GR/g) was significantly lower ($p=0.0278$) in liver samples, but significantly higher ($p=0.0034$) in brain samples compared with controls. Activity of GR/g in kidney was not significantly affected by diet or injury.

Differences in manganese superoxide dismutase activity per gram of tissue (MnSOD/g) were not significant among the diet and injury status of the four dietary groups in any of the tissues assayed.

However, activity of copper zinc superoxide dismutase per gram of tissue (CuZnSOD/g) was significantly lower ($p=0.0391$) in kidney samples from rats fed deficient pyridoxine diets, but was significantly higher ($p=0.0390$) in brain samples from rats fed the same deficient diets compared with controls. Liver sample activity was not significantly different among all dietary groups. In terms of injury status, CuZnSOD/g activity was not significantly affected in any of the three tissues assayed.

DISCUSSION

It has already been shown that vitamin B₆ affects protein metabolism (10), and in our studies, protein levels of rats with diets deficient in vitamin B₆ were significantly reduced (data not shown). However, similar to studies by Schaeffer et al. (17), body weight of all rats remained relatively constant despite the varying amount of pyridoxine in their diets (data not shown). Therefore, to insure more stable readings, activity of the antioxidant enzymes measured were expressed per grams of tissue rather than grams of protein.

Our results have shown that there were few significant decreases in enzyme activity due to changes in pyridoxine diet or injury status. One could possibly assume that with a shortfall of protein, amounts of other proteins and enzymes could also decrease. Yet this assumption did not hold for the enzymes and proteins measured in our studies because fibrinogen, IgG, IgM, GPx, GR, MnSOD, and CuZnSOD concentrations did not decrease significantly. Instead, in tissues in which antioxidant enzyme activity was significantly affected by diet, the values were usually higher in the deficient group than in the controls.

One possible explanation for these findings is that the one week allowed for recovery from surgery may have been enough time to synthesize the enzymes and proteins listed above. In addition, a previous study by Black et al. showed that rats with pyridoxine-deficient diets maintained a vitamin B₆ reserve in their gastrocnemius muscle (18). Therefore, although the plasma revealed a pyridoxine deficiency, the rats may have been relying on this storage to provide tissues with enough pyridoxine to help synthesize the needed enzymes and proteins to respond to the stress of the injury induced.

Albumin levels, on the other hand, showed a significant decrease both in the vitamin B₆ group and the injured group. Albumin in plasma was affected by pyridoxine deficiency, but the reaction of albumin as an acute-phase protein following injury was not impaired.

In conclusion, pyridoxine deficiency did not significantly affect the rat's response to surgically-induced wound. The antioxidant status of the tissues examined in the rats remained at control levels or higher, and the immunoglobulins seemed to be unaffected by the pyridoxine deficiency. Also, the acute-phase protein response to injury was not adversely affected by the pyridoxine deficiency. More research is needed to gain a better understanding of the effects of vitamin B₆ in the realm of wound healing.

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TABLE 1

Effects of Vitamin B₆ Status and Injury on Plasma
Acute Phase Protein Concentrations¹

<u>Vitamin B₆</u>		<u>Fibrinogen</u> ²	<u>Albumin</u> ^{3,4}
0 mg/kg	Uninjured	6.2±3.2	5.8±0.5
	Injured	11.2±1.8	4.8±0.3
0.25 mg/kg	Uninjured	13.4±2.1	5.6±0.2
	Injured	9.1±3.3	4.8±0.2
1.0 mg/kg	Uninjured	11.0±1.6	6.8±0.4
	Injured	12.4±0.6	6.8±0.6
7.0 mg/kg	Uninjured	10.5±1.6	7.9±0.2
	Injured	10.1±2.5	5.9±0.1

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¹Data expressed as mean ±S.E. (n=3)²Values represent arbitrary units/dl³Values represent g/dl⁴Significant effect of diet and injury at p<0.05

TABLE 2

Summary of Plasma Immunoglobulin Titers in Injured and Uninjured Rats
fed Pyridoxine-deficient diets.

<u>Diet</u>		<u>IgG</u>	<u>IgM</u>
0 mg	Uninjured	1:128	1:4
	Injured	1:128	1:4
0.25 mg	Uninjured	1:256	1:4
	Injured	1:256	1:4
1.0 mg	Uninjured	1:128	1:4
	Injured	1:128	1:2
7.0 mg	Uninjured	1:128	1:4
	Injured	1:128	1:4

TABLE 3

Effect of Vitamin B₆ Status and Injury on Tissue Antioxidant Enzymes¹Vitamin B₆ Diet

	<u>0 mg/kg</u>	<u>0.25 mg/kg</u>	<u>1.0 mg/kg</u>	<u>7.0 mg/kg</u>
<u>Glutathione Peroxidase</u>				
Liver ³				
Uninjured	105.6±9.9	82.6±4.0	100.4±15.9	89.3±2.3
Injured	99.9±10.0	67.0±5.7	87.9±3.7	85.2±5.2
Kidney				
Uninjured	11.7±0.7	13.4±0.9	16.0±1.4	14.7±0.8
Injured	14.1±1.1	10.4±2.0	14.0±0.9	13.5±0.5
Brain ⁴				
Uninjured	0.92±0.01	0.80±0.06	0.88±0.05	0.88±0.14
Injured	0.85±0.06	0.72±0.02	0.83±0.04	0.64±0.08

Glutathione Reductase

Liver ³				
Uninjured	11.2±1.0	9.7±0.4	10.1±0.9	12.5±1.4
Injured	8.3±0.05	9.2±2.1	11.9±0.3	12.4±3.7
Kidney				
Uninjured	12.8±0.7	11.3±1.5	13.1±1.2	13.1±1.1
Injured	12.5±0.5	9.2±2.1	11.9±0.3	12.4±3.7
Brain ³				
Uninjured	1.09±0.13	1.21±0.01	1.42±0.07	0.65±0.21
Injured	1.34±0.04	1.30±0.08	1.35±0.08	0.97±0.25

TABLE 3 Continued

<u>Mn SOD²</u>	Uninjured	532±109	735±154	875±79	840±204
	Liver Injured	766±234	585±38	540±38	692±70
	Uninjured	436±12	472±34	390±101	408±28
Kidney	Injured	559±53	312±47	401±75	433±56
	Uninjured	131±3	142±3	139±15	145±21
	Brain Injured	129±12	150±14	127±11	118±9
<u>CuZn SOD²</u>	Uninjured	5480±791	4935±1296	4578±1001	4428±43
	Liver Injured	4996±669	3598±702	5442±237	4975±220
	Uninjured	1075±40	1118±250	1706±177	1146±126
Kidney ³	Injured	1071±207	1398±308	1548±88	1255±19
	Uninjured	437±16	365±37	348±46	371±26
	Brain ³ Injured	469±16	386±0.3	323±67	349±50

¹Date expressed as mean ±S.E. (n=3)
²Superoxide dismutase
³Significant effect of diet at p<0.05
⁴Significant effect of injury at p<0.05

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