Dose-Response and Time-Response Study of Erythrocytosis in Rats after Intrarenal Injection of Nickel Subsulfide* †

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ABSTRACT

Erythrocytosis was induced in Fischer rats by intrarenal (ir) injection of nickel subsulfide (Ni₃S₂) in dosages ranging from 0.6 to 10 mg per rat. Measurements of blood packed cell volume (PCV) became increased by one month after ir injection of Ni₃S₂; reached maximum values at approximately two months; and gradually returned to control values by seven months. The duration and magnitude of erythrocytosis were related to the dosage of Ni₃S₂. Increases in hemoglobin concentrations and erythrocyte counts (RBC) in Ni₃S₂-treated rats were consistently proportional to blood PCV values. Blood hemoglobin concentration was 24.9 ± 1.2 g per dl at two months after bilateral ir injection of Ni₃S₂ (10 mg per rat), versus 15.8 ± 0.5 g per dl in saline-injected controls (P < 0.001). No significant changes occurred in leukocyte or platelet counts of Ni₃S₂-treated rats. Autopsy of rats killed two months after ir injection of Ni₃S₂ showed marked erythroid hyperplasia of bone marrow and fibrotic needle tracts in renal parenchyma with localized deposits of particles of Ni₃S₂. In contrast to the erythrocytosis induced by ir injection of Ni₃S₂, administration of Ni₃S₂ by intramuscular (im) injection (10 mg per rat) had no significant effect upon blood PCV, RBC or hemoglobin values or upon morphology of bone marrow.

Introduction

Jasmin and Solymoss₇ discovered that administration of nickel subsulfide, Ni₃S₂, to rats by intrarenal (ir) injection induces pronounced erythrocytosis. They observed 1.5-fold increase in blood erythrocyte count and 2.4-fold increase in body erythrocyte mass at five months after an intrarenal injection of Ni₃S₂ in dosage of 10 mg per rat. Jasmin and Solymoss₇ found that Ni₃S₂-induced erythrocytosis is not attended by alteration of erythrocyte 2,3-diphosphoglycerate levels, and they speculated that the erythrocytosis may be mediated by increased production of erythropoietin.
The present study was undertaken in order to identify the optimal experimental conditions for investigations of the mechanisms of Ni$_3$S$_2$-induced erythrocytosis. The goals of this study were (1) to establish the dose-response and time-response relationships for Ni$_3$S$_2$-induced erythrocytosis in rats and (2) to elucidate the pathological reactions that occur in Ni$_3$S$_2$-treated rats at the time of maximal erythrocytosis.

Materials and Methods

The first experiment used 67 female rats of the Fischer-344 strain,* approximately two months old at the time of injection (body weight = 180 g, SD ± 13). The rats were housed in stainless-steel cages and were fed Purina laboratory rat chow and water ad libitum. The rats were randomly distributed into three groups, A, B and C, comprising 20, 21 and 26 rats, respectively. Each rat in Group A was anesthetized with diethyl ether, and the right kidney was exposed by a subcostal lumbar incision. By use of a one ml tuberculin syringe with 25 gauge needle, 0.2 ml of sterile NaCl solution (140 mmole per liter) was injected into the upper and the lower poles of the kidney. The musculature was sutured with silk, and the skin incision was closed with surgical clips. Each rat in Group B was treated similarly but was given injections of 0.2 ml of sterile NaCl solution containing 5 mg of Ni$_3$S$_2$ dust† into the upper and the lower poles of the right kidney, amounting to a total intrarenal dosage of 10 mg of Ni$_3$S$_2$. Each rat in Group C was given a single injection of 10 mg of Ni$_3$S$_2$ dust suspended in 0.4 ml of sterile NaCl solution deep into the lateral musculature of the right thigh.

At one week after the injection and at monthly intervals for seven months, a blood sample (150 to 200 µl) was obtained from each rat's tail by use of capillary tubes that contained dried sodium heparinate (100 units per tube). Measurements of blood hemoglobin concentration, erythrocyte count (RBC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and leukocyte count (WBC) were performed in duplicate by use of an automated hemocytometer (Model S). Blood platelets were counted by phase-contrast microscopy.

At two months after the injections, ten rats in Group A, six rats in Group B and four rats in Group C were killed, and complete autopsies were performed according to the protocol of Arcos et al.1 The kidneys and thigh muscles were sectioned through the injection sites, and mid-portions of the femoral bones were decalcified for bone marrow examinations. Erythrocyte mass was determined immediately before sacrifice of four rats in Group A and four rats in Group B. For measurements of erythrocyte mass, these rats were anesthetized with diethyl ether, and the left renal vein was exposed by mid-abdominal incision. Into the renal vein was injected 0.5 ml of blood from healthy Fischer rats that had been labeled with Na$_2^{51}$CrO$_4$ by the method of Sutherland et al.14 After five minutes, a blood sample (3 ml) was obtained by cardiac puncture. $^{51}$Cr radioactivity in the blood sample was measured by use of a well-type gamma counter, and blood

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* Charles River Breeding Laboratories, North Wilmington, MA.
† The Ni$_3$S$_2$ dust was donated by Dr. Louis Renzoni, International Nickel Company, Ltd., Toronto, Canada. The median particle diameter of the Ni$_3$S$_2$ dust was 1.4 microns, based upon measurements by electron microscopy. The Ni$_3$S$_2$ dust contained 72 percent Ni and 26 percent S by weight. The Ni$_3$S$_2$ dust was analyzed for Al, Co, Cu, Cr, Fe and Mn by emission spectroscopy; contamination by these metals was less than 0.01 percent by weight. The Ni$_3$S$_2$ dust did not contain detectable NiO or NiS, based upon x-ray diffraction analyses.

† Coulter Electronics, Hialeah, FL.
TABLE I

Effects of Intrarenal or Intramuscular Administration of Ni₃S₂ upon Blood Packed Cell Volume (PCV) in Female Rats

<table>
<thead>
<tr>
<th>Ni₃S₂ Dosage (mg/rat)</th>
<th>Blood PCV at Intervals after Injection of Ni₃S₂*</th>
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<tbody>
<tr>
<td></td>
<td>1 wk</td>
</tr>
<tr>
<td>A†</td>
<td>0</td>
</tr>
<tr>
<td>[20]</td>
<td>[20]</td>
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<tr>
<td>B‡</td>
<td>10</td>
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<td>[21]</td>
<td>[21]</td>
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<tr>
<td>C§</td>
<td>10</td>
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<tr>
<td>[26]</td>
<td>[26]</td>
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</tbody>
</table>

*Packed cell volume (PCV) measurements are expressed as liter/liter; each value is the mean ± standard deviation; the number of rats studied at each interval is indicated in italics within brackets.
†Control rats (Group A) were given intrarenal injections of 0.2 ml of NaCl solution into the upper and lower poles of the right kidney.
‡Rats in Group B were given intrarenal injections of 0.2 ml of NaCl solution containing 5 mg of Ni₃S₂ dust into the upper and lower poles of the right kidney.
§Rats in Group C were given an intramuscular injection of 0.4 ml of NaCl solution containing 10 mg of Ni₃S₂ dust into the right thigh.
‡P < 0.001 vs the corresponding value in control rats (Group A), computed by "t" test.⁷
§In Group C, 2 rats died during the 5th month and 7 rats died during the 6th month, owing to sarcomas (rhabdomyosarcomas or fibrosarcomas) that developed at the site of injection of Ni₃S₂.¹³
after the injection, and gradually diminished thereafter. At six and seven months after injection, mean values for blood PCV of rats in Group B did not differ significantly from the corresponding values of control rats in Group A. No significant changes were found in mean values for blood PCV of rats in Group C at any time after intramuscular injection of NiS2.

Hematological parameters for rats in Groups A, B and C at two months after injection are summarized in Table II. Hemoglobin concentrations and erythrocyte counts of rats in Group B were increased 1.3 and 1.4-fold, respectively, compared to control rats in Group A. In contrast, hemoglobin concentrations and erythrocyte counts of rats in Group C were not significantly different from the values for control rats in Group A. No significant differences in mean values for blood MCV, MCH, MCHC, WBC or platelet counts were found in rats from Groups A, B and C at any time during the seven months of observation. As indicated in Table III, the mean value for erythrocyte mass in four rats from Group B at two months after injection was increased 2.6-fold in comparison to the mean value in four control rats (Group A).

At two months after injection, bone marrow of rats in Group B was markedly hyperplastic, with striking predominance of erythroid cells, whereas the cellularity of bone marrow of rats in Groups A and C was normal. Kidneys of rats in Group A showed focal fibrosis along narrow needle tracts where NaCl solution had been injected. Kidneys of rats in Group B showed distinct bands of fibrosis that extended from the cortex into the medulla.
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TABLE IV
Dose-response Relationship for the Effect of Intrarenal Administration of Ni3S2 upon Blood Packed Cell Volume (PCV) in Male Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Ni3S2 Dosage (mg/rat)</th>
<th>Blood PCV at Intervals after Injection of Ni3S2*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 mo†</td>
</tr>
<tr>
<td>D#</td>
<td>0</td>
<td>0.40±0.02</td>
</tr>
<tr>
<td>E†</td>
<td>0.6</td>
<td>0.41±0.01</td>
</tr>
<tr>
<td>F†</td>
<td>1.2</td>
<td>0.40±0.02</td>
</tr>
<tr>
<td>G‡</td>
<td>2.5</td>
<td>0.38±0.02</td>
</tr>
<tr>
<td>H‡</td>
<td>5.0</td>
<td>0.40±0.02</td>
</tr>
</tbody>
</table>

*Packed cell volume (PCV) measurements are expressed as liter per liter; each value is the mean ± standard deviation.
†Blood was collected immediately prior to the intrarenal injection.
#Control rats (Group D) were given an intrarenal injection of 0.2 ml of NaCl solution into the upper pole of the right kidney.
‡Ni3S2-treated rats (Groups E, F, G and H) were given an intrarenal injection of 0.2 ml of NaCl solution containing Ni3S2 dust at the specified dosage into the upper pole of the right kidney.
§P < 0.05 vs the corresponding value in control rats (Group D) computed by "t" test.17
¶P < 0.001 vs the corresponding value in control rats (Group D) computed by "t" test.17

along needle tracts where Ni3S2 dust had been injected. Large particles of Ni3S2 were predominantly extracellular, and small particles of Ni3S2 were frequently present within phagocytes. Inflammatory and fibrotic changes were limited to areas of the kidneys that were immediately adjacent to needle tracts where Ni3S2 dust had been injected. Hydronephrosis was not present, and neoplastic tissues were not identified.

Kidneys of rats in Group C were normal. Muscle at the site of injection of Ni3S2 in rats in Group C revealed focal necrosis of muscle; infiltration by lymphocytes and histiocytes, and fibroblastic and angioblastic proliferation. Black particles of Ni3S2 were present in extracellular spaces and within macrophages at the site of intramuscular injection. Histological examinations of other organs and tissues of rats in Groups A, B and C were generally unremarkable. However, in spleens of rats in Group B, there was relative diminution or complete absence of hemosiderin and giant cells within the red pulp.

Measurements of blood PCV in the second experiment (dose-response study) are listed in Table IV. Rats in Group E that received the lowest dose of Ni3S2 (0.6 mg) developed significant increases in mean values for blood PCV at one to four months after injection, compared to the values for saline-injected control rats (Group D). At one and two months after intrarenal injections of Ni3S2 in Groups E to H, no clear-cut relationships were observed between the dosages of Ni3S2 (0.6 to 5 mg) and the mean values for blood PCV. However, at three, four, five and six months after the intrarenal injections, the mean values for blood PCV were increased in correlation with increasing dosages of Ni3S2. At seven months after the injections, mean values for blood PCV in Groups G and H (2.5 and 5.0 mg Ni3S2) did not differ significantly from the mean value for control rats (Group D). No significant differences in mean values for leukocyte and platelet counts or erythrocyte indices (MCV, MCH, MCHC) were found between Groups D, E, F, G or H during seven months of observation. Hemoglobin concentrations and eryth-
TABLE V
Effects of Bilateral and Unilateral Intrarenal Administration of Ni$_3$S$_2$ upon Blood Packed Cell Volume (PCV) in Female Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Ni$_3$S$_2$ Dosage (mg/rat)</th>
<th>Blood PCV at Intervals after Injection of Ni$_3$S$_2$*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 mo†</td>
</tr>
<tr>
<td>I†</td>
<td>(N=8)</td>
<td>0</td>
</tr>
<tr>
<td>J‡</td>
<td>(N=7)</td>
<td>10</td>
</tr>
<tr>
<td>K§</td>
<td>(N=8)</td>
<td>5</td>
</tr>
</tbody>
</table>

*Packed cell volume (PCV) measurements are expressed as liter per liter; each value is the mean ± standard deviation.
†Blood was collected immediately prior to the intrarenal injections.
‡Control rats (Group I) were given intrarenal injections of 0.2 ml of NaCl solution into the upper and lower poles of the right and left kidneys.
§Rats in Group J were given intrarenal injections of 0.2 ml of NaCl solution containing 2.5 mg of Ni$_3$S$_2$ into the upper and lower poles of the right and left kidney.
#Rats in Group K were given intrarenal injections of 0.2 ml of NaCl solution containing 2.5 mg of Ni$_3$S$_2$ into the upper and lower poles of the right kidney.
§P < 0.001 vs the corresponding value in control rats (Group I), computed by "t" test.17

cyte counts in Groups D to H were consistently proportional to blood PCV values.

Measurements of blood PCV in the third experiment are given in table V. The mean values for blood PCV in Group J at one to four months after bilateral intrarenal injection of 5 mg of Ni$_3$S$_2$ (total dosage = 10 mg per rat) were not significantly different from the corresponding values that were obtained after unilateral intrarenal injection of 5 mg of Ni$_3$S$_2$ (Group K). At two months after bilateral injection of Ni$_3$S$_2$ (Group J), the mean concentration of blood hemoglobin was 24.9 ± 1.2 g per dl, versus corresponding mean values of 15.8 ± 0.5 g per dl in control rats (Group I, P < 0.001), and 23.5 ± 1.3 g/dl in rats with unilateral injection of Ni$_3$S$_2$ (Group K, P < 0.05). At two months after bilateral intrarenal injection of Ni$_3$S$_2$ (Group J), the mean erythrocyte count was 15.3 (± 0.6) × 10$^{12}$ per liter, versus corresponding mean values of 7.7 (± 0.3) × 10$^{12}$ per liter in control rats (Group I, P < 0.001), and 14.3 (± 0.5) × 10$^{12}$ per liter in rats with unilateral injection of Ni$_3$S$_2$ (Group K, P < 0.01). No significant differences in mean values for leukocyte and platelet counts or erythrocyte indices (MCV, MCH, MCHC) were observed between Groups I, J or K during four months of observation.

Discussion

The present investigation confirmed the finding of Jasmin and Solymoss7 that intrarenal injection of Ni$_3$S$_2$ induces marked erythrocytosis in rats. In addition, this study showed that maximum erythrocytosis occurs at approximately two months after the intrarenal injection, and that the duration and intensity of erythrocytosis are related to the dosage of Ni$_3$S$_2$. Significant erythrocytosis was observed after intrarenal injection of Ni$_3$S$_2$ at the lowest dosage that was tested (0.6 mg per rat).

This study also demonstrated that intramuscular injection of Ni$_3$S$_2$ to rats does not cause erythrocytosis. Sunderman et al12 investigated $^{63}$Ni-kinetics after intraperitoneal injection of $^{63}$Ni$_3$S$_2$ in rats, and found that $^{63}$Ni(II) is slowly mobilized from the site of injection and excreted in the urine. After intraperitoneal injection of 1.2 mg of $^{63}$Ni$_3$S$_2$, urinary excretion of $^{63}$Ni(II) averaged 37 ± 2 percent of the dose within two weeks and 67 ± 2 percent of the dose within two months.12 From these data, it is clear that
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*im* injection of Ni$_3$S$_2$ results in sustained exposure to the kidneys to Ni(II). The failure of erythrocytosis to develop after *im* injection of Ni$_3$S$_2$ is consistent with previous reports that chronic oral or parenteral administration of Ni(II) to rats does not produce erythrocytosis, although administration of Co(II) causes marked stimulation of erythropoiesis under similar experimental conditions.$^5,10$

Toyama and Mitus$^{16}$ reported that unilateral hydronephrosis induces erythrocytosis in rabbits. In the present study, histological examination of the kidneys of rats that were killed at the time of maximum erythrocytosis showed localized scarring, but did not reveal generalized pathological changes. There was no evidence of hydronephrosis. Therefore, Ni$_3$S$_2$-induced erythrocytosis is probably not mediated by increased hydronephrotic pressure.

Mirand et al$^9$ detected increased concentrations of erythropoietin in plasma of rats with renal adenocarcinomas that were induced by dimethylnitrosamine. Since Jasmin$^6$ observed renal adenocarcinomas in rats at eight to 12 months after intrarenal injection of Ni$_3$S$_2$, the present authors considered the possibility that Ni$_3$S$_2$-induced erythrocytosis might be a manifestation of erythropoietin production by renal neoplasms. This possibility does not seem tenable, owing to the relatively rapid development of erythrocytosis, and the absence of histological evidence of neoplasia in kidneys that were examined at two months after intrarenal injection of Ni$_3$S$_2$.

Swierenga$^{15}$ found that Ni$_3$S$_2$ profoundly inhibits glyceraldehyde-3-phosphate dehydrogenase activity in tissue cultures of rat embryo muscle cells, whereas NiCl$_2$ and NiSO$_4$ cause only slight inhibition of this important glycolytic enzyme. Inhibition of glyceraldehyde-3-phosphate dehydrogenase results in a shift of oxidative metabolism to the alternative hexosemonophosphate pathway.$^{15}$ Such an alteration of energy metabolism in renal cells might mimic renal cell hypoxia, which has been shown to stimulate erythropoietin production.$^3,4$

The findings of Jasmin and Solymoss$^7$ and the results of the present study demonstrate that intrarenal injection of Ni$_3$S$_2$ is a convenient, effective and reproducible experimental system for induction of erythrocytosis in rats. In the present study, the concentration of blood hemoglobin reached 25 g per dl at two months after bilateral intrarenal injections of 5 mg of Ni$_3$S$_2$ (a total dosage of 10 mg of Ni$_3$S$_2$, equivalent to 7.2 mg of Ni). For comparison, Korst and Bethell$^8$ found that the concentration of blood hemoglobin reached 18.5 g per dl after daily intraperitoneal injections of two mg of Co(II) to rats for 25 successive days (a total dosage of 50 mg of Co). Studies are currently in progress in our laboratory to establish (1) the distribution and rates of excretion of$^{63}$Ni after intrarenal injection of$^{63}$Ni$_3$S$_2$ in rats; (2) whether or not erythrocytosis is induced in rats by intrarenal injection of other insoluble nickel compounds; (3) whether or not there are differences among rat strains in susceptibility to Ni$_3$S$_2$-induced erythrocytosis, such as have been reported for susceptibility to Ni$_3$S$_2$-tumorigenesis$^2$; and (4) whether or not increased levels of erythropoietin can be detected in plasma and urine of rats after intrarenal injection of Ni$_3$S$_2$.

**Acknowledgments**

The authors are grateful to Dr. Gaëtan Jasmin, Dr. Bela Solymoss, Dr. Allan J. Erslev, Dr. Torgny Fredrickson and Mr. Sidney Hopfer for valuable advice and assistance; to Mr. John Mitchell for skilful performance of intrarenal injections and bleeding of rats; to Mrs. Patricia Allpass for preparation of histological sections; and to Mr. George Kalache, Miss Sophia Panek, Mr. James Erickson and Mr. Theodore McIntosh for technical assistance with hematological tests.
References


