The Influence of Cobalt and Sheep Erythropoietin on Radioactive Iron Incorporation in RBC of Starved Intact and Nephrectomized Rats

By Neil P. Sanzari and James W. Fisher

A review of the recent literature concerning the site of erythropoietin production unmistakably reveals the existence of conflicting views as to the role of the kidney in erythropoiesis. Experimental observations from several laboratories indicate that the kidney is a primary source of at least one erythropoietic hormone. However, there is good evidence for the existence of extrarenal erythropoietic factors. It has also been postulated that the kidney is "permissive" rather than "active" in erythropoiesis.

Most of the controversial evidence has been obtained with an assay method involving the use of a nephrectomized donor animal receiving an erythropoietic stimulus and its plasma subsequently assayed in an intact recipient animal. The presence of the kidney in the intact recipient makes it exceedingly difficult to interpret such results.

In the studies to be reported we have attempted to clarify the response of nephrectomized animals to erythropoietic stimuli by employing a "direct" assay method which involves stimulating and measuring erythropoietic activity in the same animal. The starved rat was selected as the test animal because of its increased sensitivity to erythropoietic stimuli. We have also found that the starved rat withstands the stress of bilateral nephrectomy better than other assay animals. The erythropoietic response of the starved-nephrectomized rat to sheep erythropoietin and cobalt was investigated. Other studies are reported concerning (1) the influence of nephrectomy, sheep erythropoietin and cobalt on plasma iron, and (2) the effects of nephrectomy on total blood volume.

Materials and Methods

All animals employed in this study were male Holtzman albino rats. The procedure of Fried and co-workers was followed for the radioactive iron incorporation in RBC studies. The age of all rats used ranged from 45-50 weeks. At the termination of the experiments, which lasted for 5 days, the mean body weight for all animals studied was 309 ± 1.38 Gm. All rats were starved throughout the 5-day assay period, water being permitted ad libitum. After 48 hours of starvation the animals to be nephrectomized were lightly anesthetized.

From the Department of Pharmacology, University of Tennessee Medical Units, Memphis, Tenn.

This investigation was supported by USPHS Grant A-2973(C3) from the Institute of Arthritis and Metabolic Diseases and USPHS Research Career Development Award Grant GM-K3-5990-C2.

Part of the work reported in this paper was presented as one author's (N. P. S.) partial fulfillment of the requirements for a Master of Science Degree in Pharmacology. An abstract of this work was also presented at Fed. Proc. 24:70, 1962.

Submitted Nov. 6, 1962; accepted for publication Jan. 18, 1963.

729

Blood, Vol. 21, No. 6 (June), 1963
with ether and the kidneys removed through bilateral lumbar incisions. Peritoneal lavage tubes were implanted in the anterior abdominal wall via a modification of the technic of Kolf and Page. Immediately following nephrectomy, one-third of the total dose of either cobalt or sheep erythropoietin was injected subcutaneously. The second one-third of the total dose was administered approximately 4 hours before two successive 1-hour peritoneal lavages which was 24 hours after nephrectomy. A polyethylene nasal catheter was used for introducing Kolf rinsing solution and for removing the exudate. Thirty hours after nephrectomy the final one-third of the total dose was administered at the same time that 1 ml. of saline containing one pCi Fe⁵⁹ citrate was injected intravenously via the tail vein. Standard samples were prepared for later counting. Sixteen hours later, 1 ml. of blood was drawn via cardiac aspiration, delivered into an oxalated test tube, and the Fe⁵⁹ incorporation in red cells was calculated according to the following formula:

\[
\text{Fe}^{59} \text{ uptake} = \frac{\text{net counts per ml. blood x derived bld. vol. in per cent x body wt.}}{\text{net counts injected}}
\]

The total circulating red cell volume was determined on each rat by the Cr⁵¹ tagged red cell method and the derived blood volume was used in the above formula for calculating Fe⁵⁹ incorporation in RBC. All animals in which blood volume was determined were treated in the same manner as described for the direct assay procedure with the exception that the Fe⁵⁹ was deleted and Cr⁵¹ RBC volumes determined on the 5th day as follows: Two-tenth ml. of a Cr⁵¹ tagged red cell suspension was injected into the saphenous vein while the animal was lightly anesthetized with ether, and allowed to mix for 10 minutes, at which time blood was removed via cardiac aspiration and delivered into a heparinized tube. One ml. of this blood was counted with the use of a scintillation counter. Microhemocrits were determined on the blood sample with heparinized capillary tubes. The blood volumes were derived from the total circulating red cell volume by the following formula:

\[
\text{Blood volume (ml./100 Gm.)} = \frac{\text{RBC volume (ml./100 Gm.)}}{\text{whole body hematocrit}} \times 100
\]

Plasma iron was determined by the method of Peters et al. The blood urea nitrogen was measured by direct nesslerization and plasma potassium by flame photometry. Step III erythropoietin from plasma of phenylhydrazine-treated sheep was supplied by the USPHS Hematology Study Section. The erythropoietin activity was approximately 5 cobalt units per mg. protein. The sheep erythropoietin was diluted with 0.85 per cent saline before injection and kept frozen when not being used. Analytical grade cobaltous chloride hexahydrate with maximum impurity for lead of 0.005 per cent was dissolved in 0.85 per cent saline before injection. The technic of analysis of variance was used in determining significant differences between experimental groups.

**RESULTS**

1. **Influence of Nephrectomy on Total Circulating Red Cell Volume and Blood Volume in Starved Rats**

Table 1 shows the results of Cr⁵¹ circulating red cell volume and blood volume in two experiments. The mean red cell volume in nephrectomized rats (2.19 ± .11 ml./100 Gm.) was significantly less than that of intact controls (2.51 ± .06 ml./100 Gm.). When blood volumes were calculated from these data, the mean value for nephrectomized rats (4.86 ± .24 ml./100 Gm.) was found to be significantly less than that of intact controls (5.64 ± .02 ml./100 Gm.). Since lower blood volume values were found in nephrectomized rats, the values of .0564 for intact rats and .0486 for nephrectomized
Table 1.—Mean Circulating RBC and Blood Volumes in Intact and Nephrectomized Rats

<table>
<thead>
<tr>
<th>Group*</th>
<th>Number of Animals</th>
<th>Mean RBC Volume (ml./100 Gm.)</th>
<th>Mean Blood Volume (ml./100 Gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>10</td>
<td>2.51 ± .06</td>
<td>5.64 ± .02</td>
</tr>
<tr>
<td>Nephrectomized</td>
<td>9</td>
<td>2.19 ± .11†</td>
<td>4.86 ± .24†</td>
</tr>
</tbody>
</table>

± = Standard error of the mean.
*Saline-treated.
†Difference from intact group is significant at the 1 per cent level.

rats were used for calculating Fe⁵⁹ incorporation in RBC rather than an estimated value of .05.

2. Fe⁵⁹ Incorporation in RBC of Starved-Intact and Starved-Nephrectomized Rats Exposed to Peritoneal Dialysis

As demonstrated in figures 1 and 2, starved-intact rats (n = 33) injected with saline showed a baseline iron incorporation of 12.57 ± .56 per cent. The baseline iron incorporation for starved-nephrectomized rats (n = 22) injected with saline and subjected to peritoneal dialysis was 14.65 ± .54 per cent. This slightly higher Fe⁵⁹ incorporation was not statistically significant. Preliminary experiments revealed that Fe⁵⁹ incorporation values in starved sham-operated rats exposed to peritoneal dialysis were not significantly different from those of starved non-lavaged intact rats. Therefore, the intact rats in these studies were not exposed to peritoneal dialysis or sham operation.

3. Influence of Sheep Erythropoietin on Fe⁵⁹ Incorporation in RBC of Starved-Intact and Starved-Nephrectomized Rats

In order to determine if the bone marrow of the starved-nephrectomized rat was responsive to erythropoietin itself, we compared the effects of sheep erythropoietin in starved-intact and nephrectomized rats. As seen in figure 1, total dosages of 6, 12, 24 and 36 units of sheep erythropoietin per Kg. produced a progressive increase in iron incorporation in both intact and nephrectomized rats. There was not a significant difference between the responses of the intact and nephrectomized rats to erythropoietin. Therefore, nephrectomy has not apparently interfered with the response of the erythroid tissue to erythropoietin.

4. Influence of Cobalt on Fe⁵⁹ Incorporation in RBC of Starved-Intact and Starved-Nephrectomized Rats

As indicated in figure 2, total dosages of 120 and 180 μmoles cobalt per Kg. produced significantly greater Fe⁵⁹ incorporation in starved-intact than in starved-nephrectomized rats. On the other hand, there was no significant difference between the responses to the 30 and 60 μmole dosages of cobalt in intact and nephrectomized rats. The Fe⁵⁹ incorporation values in all intact rats receiving cobalt were significantly greater than saline intact controls, with the exception of the group receiving the 30 μmole dosage. Mean Fe⁵⁹ incorporation values in nephrectomized animals receiving cobalt were not
Fig. 1.—Influence of sheep erythropoietin on radioactive iron incorporation in RBC of intact and nephrectomized rats. Each point on the curves represents 8 to 12 rats receiving erythropoietin with the exception of the intact saline-treated (n = 33) and nephrectomized saline-treated (n = 22) groups.

Fig. 2.—The influence of cobalt on radioactive iron incorporation in RBC of intact and nephrectomized rats. Each point on the curves represents 9 to 16 rats receiving cobalt with the exception of the intact saline-treated (n = 33) and nephrectomized saline-treated (n = 22) groups.
Table 2.—Mean Plasma Iron in Starved-Intact and Starved-Nephrectomized Rats Treated with Cobalt or Sheep Erythropoietin

<table>
<thead>
<tr>
<th>Total Dose Cobalt (μmoles/Kg.)</th>
<th>No. of Rats</th>
<th>Plasma Iron (μg. per cent)</th>
<th>Nephrectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td>112.3 ± 7.2</td>
<td>9</td>
</tr>
<tr>
<td>60</td>
<td>10</td>
<td>108.6 ± 6.4</td>
<td>10</td>
</tr>
<tr>
<td>120</td>
<td>10</td>
<td>98.2 ± 5.2*</td>
<td>5</td>
</tr>
<tr>
<td>180</td>
<td>8</td>
<td>93.6 ± 9.3*</td>
<td>3</td>
</tr>
</tbody>
</table>

Sheep erythropoietin (units/Kg.)

<table>
<thead>
<tr>
<th></th>
<th>No. of Rats</th>
<th>Plasma Iron (μg. per cent)</th>
<th>Nephrectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intact</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>112.1 ± 3.5</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>111.7 ± 5.1</td>
<td>4</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>115.6 ± 24.5</td>
<td>5</td>
</tr>
<tr>
<td>36</td>
<td>5</td>
<td>114.6 ± 17.5</td>
<td>5</td>
</tr>
</tbody>
</table>

± = Standard error of the mean.

*Significantly different from respective saline control at the 5 per cent level.
†Significantly different from their respective intact control at the 5 per cent level.

significantly different from nephrectomized controls. Therefore, it may be concluded that nephrectomy results in a significant reduction in the erythropoietic response to cobalt injections.

5. Influence of Nephrectomy, Sheep Erythropoietin and Cobalt Administration on Plasma Iron

Table 2 indicates that nephrectomy alone results in a significant reduction in plasma iron in the starved rat. The mean plasma iron value of untreated nephrectomized animals (92.7 ± 3.5 μg. per cent) was significantly less than that of intact controls (112.3 ± 7.2 μg. per cent). Both starved-intact and -nephrectomized rats receiving total dosages of 60, 120 and 180 μmoles cobalt per Kg. showed a progressive decline in plasma iron. There was a more pronounced reduction in plasma iron in nephrectomized rats receiving cobalt than that seen in the intact rats. The plasma iron values in all nephrectomized groups were significantly less than their respective intact controls. There was no significant change in the plasma iron of either starved-intact or starved-nephrectomized rats receiving 12, 24 or 36 units of sheep erythropoietin per Kg. Therefore, the erythropoietic response of nephrectomized rats receiving erythropoietin could not be the result of higher plasma Fe59 specific activity due to a reduction in plasma iron.

6. Plasma BUN and Potassium Values in Starved-Intact and Starved-Lavaged-Nephrectomized Rats

Since the toxic manifestations of nephrectomy have been postulated to modify the response of nephrectomized animals to erythropoietic stimuli, it seemed important to determine the degree of uremia present in lavaged-nephrectomized rats used in our studies.

As indicated in table 3, the mean plasma BUN value for 5-day starved
Table 3.—Influence of Peritoneal Dialysis on Plasma BUN and Potassium in Intact and Lavaged-Nephrectomized Rats

<table>
<thead>
<tr>
<th>Group (starved)</th>
<th>BUN (mg. per cent)</th>
<th>Potassium (mEq./L.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>13.1 ± 1.4</td>
<td>5.5 ± 0.50</td>
</tr>
<tr>
<td>Nephrectomized</td>
<td>63.8 ± 1.5</td>
<td>6.3 ± 0.35</td>
</tr>
</tbody>
</table>

± = Standard error of the mean.
*The plasma BUN and potassium determinations were made in two separate experiments with five animals in each group.
†Significantly different from intact group at the 1 per cent level.

rats 48 hours after nephrectomy, exposed to peritoneal dialysis, was significantly higher than that of saline-treated intact rats starved for 5 days. It is interesting to note than the BUN is elevated in nephrectomized rats despite peritoneal dialysis. Apparently this elevation in BUN has not significantly modified the response of the nephrectomized rat to erythropoietin.

Table 3 also indicates that the mean plasma potassium values in starved-nephrectomized rats were slightly greater than those in intact controls. However, this slight increase in plasma potassium was not statistically significant. Although the data are not available in these studies, it is suggested that peritoneal dialysis has prevented an excessive rise in plasma potassium.

DISCUSSION

Sheep erythropoietin injections into nephrectomized rats resulted in an erythropoietic response which was comparable to that seen in intact rats. This finding is important in that it establishes the fact that the erythroid tissue of nephrectomized rats is capable of eliciting a normal ferrikinet response to erythropoietin.

It has been suggested that the toxic manifestations of nephrectomy, including uremia, cause the erythroid tissue to become refractory to erythropoietin. Therefore, a further significance of the above finding is that the uremia which occurred in the nephrectomized animal, indicated by an increase in plasma BUN, did not modify the capacity of the bone marrow to respond to erythropoietin. Perhaps when the uremia is more severe or of a longer duration, the erythroid tissue may become less responsive. Our failure to find a significant elevation in plasma potassium in dialyzed animals indicates that hyperkalemia, which is another toxic manifestation of nephrectomy, has been prevented.

Assay methods involving the use of an intact-recipient rat have produced conflicting data. While some workers have been unable to demonstrate a significant increase in plasma erythropoietin titers in nephrectomized donors, others have reported significant increases in erythropoietin levels in plasma from nephrectomized donors when such plasma is assayed in intact recipients. The direct-assay method used in the present studies has eliminated any possible renal influence of a recipient animal and allows a more clear interpretation of the role of the kidney in erythropoiesis. Even though the nephrectomized rat is capable of responding to erythropoietin, removal of the kidneys has markedly reduced the erythropoietic effects of cobalt. The slight
response to the 120-μmole dose of cobalt in nephrectomized rats may be due to a change in specific activity of radioactive iron in plasma resulting from the reduction in plasma iron produced by cobalt. This finding may also be a manifestation of another extrarenal effect of cobalt and support the postulate that erythropoietic stimuli cause the discharge of multiple target organ hormones which either alone or in summation could produce an erythropoietic effect. The suggestion by Osnes that a kidney factor permits metabolic responsiveness of the erythroid tissue to erythropoietin is not a tenable hypothesis in view of the finding that the nephrectomized animal is capable of responding to erythropoietin. Recently, other workers have provided more direct evidence that the kidney elaborates an active erythropoietic factor by demonstrating the production of erythropoietin by an isolated perfused kidney. Therefore, there is little doubt that the kidney plays an "active" role in erythropoiesis and the existence of a "permissive" renal factor seems unlikely.

The observation in this report showing that nephrectomy reduces total circulating blood volume, primarily through a reduction in RBC volume, is particularly significant since it demonstrates that the use of a value of 5 per cent of body weight as an estimate of blood volume in the rat may lead to misleading radioactive iron incorporation values. Several factors may account for the decrease in total red cell and blood volumes following nephrectomy. The removal of a moderate amount of blood contained in the kidneys, along with the loss of vascular space occupied by the kidneys, could partially account for the reduction in blood volume. Another contributing factor could be an acceleration in the rate of destruction of red cells due to the release of one or more hemolytic factors. Finally, the removal of the kidneys, a primary source of erythropoietin, would lead to a marked decrease in the production of red blood cells by the erythroid tissue.

The finding of a slightly higher baseline iron incorporation in nephrectomized rats than in intact controls may be related to our finding that plasma iron is significantly less in nephrectomized than intact rats. Such a reduction in plasma iron may lead to a higher specific activity of iron in the nephrectomized than in the intact animal. It seems unlikely that the decrease in plasma iron following nephrectomy can be attributed to an increase in the incorporation of iron into hemoglobin of newly formed red cells because nephrectomy removes the primary source of the erythropoietic stimulus. A possible explanation for the lower plasma iron values in nephrectomized animals could be the blood lost at the time of surgery. However, the only observable blood lost was that contained in the kidneys at the time of nephrectomy. We would not expect this amount of blood loss to totally account for the reduction in plasma iron seen in the nephrectomized animals, because other investigators have shown that plasma iron concentrations in animals subjected to acute hemorrhage are not significantly reduced until 9 to 14 days after bleeding. Another possible explanation for this effect is that nephrectomy results in the diversion of plasma iron to storage. If the kidney is the sole source of the erythropoietic hormone, it is surprising that iron incorporation occurs at all in the nephrectomized animal. This finding
may be further evidence for the existence of extrarenal erythropoietic factors. On the other hand, baseline iron incorporation may be found to be further depressed in animals maintained for a longer period of time after nephrectomy.

In view of the finding that plasma iron is not altered in either the intact or nephrectomized rat after erythropoietin administration, and since cobalt exerts its erythropoietic effect through production of kidney erythropoietin, it does not seem possible that the reduction in plasma iron produced by cobalt in intact and nephrectomized rats is due to an increased utilization of iron for hemoglobin synthesis resulting from enhanced erythropoiesis. A more plausible explanation for the influence of cobalt on plasma iron in nephrectomized and intact rats would seem to be a diversion of plasma iron to storage. At the present time we have no explanation for the more marked effect of cobalt on plasma iron in the nephrectomized rat.

**SUMMARY**

The direct-assay of cobalt and sheep erythropoietin in the nephrectomized rat revealed that removal of the kidneys reduced the erythropoietic response to cobalt but did not modify significantly the response to sheep erythropoietin. Neither sham operation nor peritoneal dialysis was observed to exert a significant influence on radioactive iron incorporation in the starved rat. A moderate elevation in plasma BUN occurred in the dialyzed nephrectomized rats but did not modify the capacity of the nephrectomized rat to respond to erythropoietin. No significant change occurred in plasma potassium in the lavaged-nephrectomized rats. Bilateral nephrectomy was found to result in a significant reduction in total circulating red cell and blood volume. Plasma iron was significantly reduced by both nephrectomy and cobalt administration, but was not significantly influenced by sheep erythropoietin. The findings reported in these studies, as well as those of other workers, leaves little doubt that the kidney plays an "active" role in erythropoiesis. The existence of a "permissive" renal erythropoietic factor does not seem likely.

**SUMMARIO IN INTERLINGUA**

Le essayage directe de cobalt e erythropoietina ovin in le nephrectomisate ratto revelava que le excision del reiies reduce le responsa erythropoietic a cobalt sed non modifica significativevemente le responsa a erythropoietina ovin. Esseva constatate que ni operationes fictitie ni dialyse peritonee exercite un significative influentia super le incorporation de ferro radioactive in le affamate ratto. Un moderate elevation del nitrogeno de urea sanguinee occurreva in le dialysate rattos post nephrectomia, sed isto non alterava le capacitate del nephrectomisate rattos de responder a erythropoietina. Nulle significative alteration occurreva in le kalium del plasma in le rattos post lavage e nephrectomia. Esseva trovate que nephrectomia bilateral resulta in un significative reduction del total erythrocytos circulante e del volumine de sanguine. Ferro de plasma esseva significativemente reducite tanto per nephrectomia como
etiam per le administration de cobalt, sed illo non esseva influentiate a grados significative per erythropoietina ovin. Le constatationes reportate in iste studios—in conjunction con illos trovate per altere autores—permitte pauc dubita que le ren ha un rolo active in le erythropoiesis. Le existentia de un "permissive" renal factor erythropoietic non es multo probabile.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of Mrs. Judy Parker and Mr. Dan Knight of the University of Tennessee Medical Units. We are also indebted to the USPHS Hematology Study Section for supplying the sheep erythropoietin used in this study.

REFERENCES


Neil P. Sanzari, B.S., M.S., Department of Pharmacology, University of Tennessee Medical Units, Memphis, Tenn.

James W. Fisher, Ph.D., Associate Professor of Pharmacology, Department of Pharmacology, University of Tennessee Medical Units, Memphis, Tenn.
The Influence of Cobalt and Sheep Erythropoietin on Radioactive Iron Incorporation in RBC of Starved Intact and Nephrectomized Rats

NEIL P. SANZARI and JAMES W. FISHER