Effects of Adding Two Forms of Supplemental Zinc to a Practical Diet on Skin Regeneration in Holstein Heifers and Evaluation of a Procedure for Determining Rate of Wound Healing

W. J. MILLER, D. M. BLACKMON, J. M. HIERS, JR., P. R. FOWLER, C. M. CLIFTON, and R. P. GENTRY
Dairy Science Department and School of Veterinary Medicine, University of Georgia, Athens

Abstract
A procedure for measuring wound healing in terms of skin regeneration following removal of biopsy sections was evaluated. The effects of adding supplemental zinc oxide and zinc sulfate to a practical diet also were studied. Eighteen Holstein heifers, averaging 5.7 months, were fed limited concentrates and Coastal Bermudagrass hay ad libitum with two-thirds of the dry matter coming from hay. On a dry basis zinc contents of the total diets were: a) control, 30 ppm; b) zinc oxide, 400 ppm; and c) zinc sulfate, 384 ppm. Weight gains, serum alkaline phosphatase, and rate of skin regeneration were not affected appreciably by the dietary treatments during the 6-wk treatment period. Calves fed the zinc supplements had somewhat higher whole blood zinc levels. The procedure for measuring rate of wound healing consisted of surgically removing diamond-shaped pieces of skin from the gluteal regions and determining length of diagonals of the unhealed area at weekly intervals. With the variance observed in this study, difference in skin regeneration rate of one to four days, from 1 to 6 wk following surgery would be sufficient to be statistically significant (P = 0.05). Two relatively untrained observers independently obtained estimates that were comparable.

Supplementation of a normal diet with zinc substantially enhanced wound healing in apparently healthy human males. Earlier studies showed that wound healing was impaired in zinc-deficient calves (1). After the animals recovered from the deficiency, rate of healing was equal to that of controls, but the effects of feeding higher than normal amounts of zinc were not investigated.

Previously an experimental wound-healing procedure was developed. It involved the surgical removal of diamond-shaped pieces of skin and measurement of the area over which new skin had not grown at time intervals (11). Data were not presented to show the extent of the variability and the effects of different factors on the variation obtained by this procedure.

The experiment herein reported was designed to determine the effects of adding a fairly high level of supplemental zinc to a practical diet on rate of wound healing, weight gains, level of blood zinc, and serum alkaline phosphatase in growing cattle. A part of the objective was to determine the influence of both a highly soluble and an insoluble form of zinc. Another major objective was to evaluate the variability of the measurements of the unhealed areas at various times after surgery and to determine the variation which might be expected when different individuals made the measurements. It was envisioned that optimal wound healing may not always coincide with optimal nutritional status, as indicated by the commonly used criteria, such as rate of gain, blood studies, etc.

Experimental Procedure
Eighteen Holstein heifer calves averaging 5.7 months of age and 139 kg in initial weight were assigned to three groups equalized on the basis of age, weight, and hair color on the gluteal region. The groups were randomly allotted to receive one of three concentrates: a) control, b) control + zinc oxide (ZnO), or c) control + zinc sulfate (ZnSO₄·H₂O). Each of the zinc compounds was added at the rate of 1,000 ppm of zinc to the concentrate, expressed on an air-dry basis. Zinc additions were added at the expense of the whole diet. The control diet consisted of the following: corn meal, 56.87%; citrus pulp, 20.0%; soybean oil meal, 20.0%; trace-mineralized salt, 1.0%; defluorinated rock phosphate, 1.8%; chlorotetracycline (110 g/kg),...
Methylparaben, 0.25 g; propylparaben, 0.15 g; sodium lauryl sulfate, 10 g; white petrolatum, 250 g; and purified water, propylene glycol, 120 g; stearyl alcohol, 250 g; and gluteal (rump) regions on both the left and right side of each calf. These areas were chosen because they are well protected against accidental injury. The wounds were made by surgically removing diamond-shaped areas of skin which had diagonals of 5.8 and 4.5 cm in length and width. In depth they extended completely through the skin and to, but not through, the underlying fascia. These wounds were permitted to heal without sutures. Wound areas were medicated daily with Hydrophilic Ointment U. S. P. XVI (15), which contains the following: methylparaben, 0.25 g; propylparaben, 0.15 g; sodium lauryl sulfate, 10 g; propylene glycol, 120 g; stearyl alcohol, 250 g; white petrolatum, 250 g; and purified water, 370 g; for a total of 1,000 g. Sulphathiazole was added at the rate of 5%.

Following surgery, the diagonals of the area over which skin was absent were measured at one day and at weekly intervals for 6 wk. This procedure was developed for a previous study (11).

Even though all the skin biopsy samples were made by the same pattern and by the same surgeon, there were small differences in the initial measurements made on the day following surgery. Since new skin regeneration is expected to proceed in a linear pattern from every side of the wound, but not at a linear rate with time, all measurements were adjusted for the small differences observed at the initial measurement period, assuming a regression coefficient of 1.0.

Blood samples were obtained four days prior to taking skin biopsy samples; at 2, 4, and 6 wk after the surgery was performed; and seven days after all the animals were returned to the control diet. Blood samples were analyzed for zinc by atomic absorption spectrophotometry (1), with nitric-perchloric-sulfuric acid wet ashing of samples. Serum alkaline phosphatase was determined (2) each time blood was collected.

Results and Discussion

Zinc contents of both the control concentrate (42 ppm) and the Coastal Bermudagrass hay (24 ppm) were apparently fairly typical of that to be expected in many practical heifer diets (Table 1) (6, 10). The level of supplemental zinc added (358 ppm of zinc oxide and 342 ppm of zinc sulfate) was far above the amount normally expected to be required for normal growth and health in heifers (8), but substantially below amounts that cause toxicity (9, 13).

The supplemental zinc did not affect rate of gain or hay consumption (Table 1). The good growth rate and high level of hay consumption for this type of diet is no doubt at least partially due to the animals being relatively small and thin for their age at the time the study was initiated.

When the heifers were fed supplemental zinc, zinc content of whole blood increased relative to that of controls (Table 1). The decline in blood zinc level of the controls from the pretreatment samples collected when the pretreatment samples were collected, the decline was somehow related to the dietary changes. The relative increase in zinc content...
TABLE 1
Zinc content, weight, age, feed consumption, blood zinc, and serum alkaline phosphatase values of animals fed the three diets

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Zinc oxide</th>
<th>Zinc sulfate</th>
<th>SEa</th>
<th>C.V. b (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc content of feeds (ppm of d. m.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentrate</td>
<td>42</td>
<td>1,153</td>
<td>1,153</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hay</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total diet</td>
<td>30</td>
<td>400</td>
<td>384</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial weight (kg)c</td>
<td>139</td>
<td>139</td>
<td>138</td>
<td>8.0</td>
<td>32.2</td>
</tr>
<tr>
<td>Initial age (days)c</td>
<td>175</td>
<td>172</td>
<td>173</td>
<td>14.7</td>
<td>20.8</td>
</tr>
<tr>
<td>Wt gain (kg/day)</td>
<td>0.94</td>
<td>1.00</td>
<td>0.97</td>
<td>0.052</td>
<td>13.1</td>
</tr>
<tr>
<td>Concentrates eaten (kg d. m./day)</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hay eaten (kg d. m./day)</td>
<td>4.1</td>
<td>4.4</td>
<td>4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood zinc (ppm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment period</td>
<td>2.17</td>
<td>2.25</td>
<td>2.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment period</td>
<td>1.48</td>
<td>2.31</td>
<td>1.86</td>
<td>0.06</td>
<td>7.2</td>
</tr>
<tr>
<td>Post-treatment period</td>
<td>1.46</td>
<td>1.79</td>
<td>1.87</td>
<td>0.08</td>
<td>11.4</td>
</tr>
<tr>
<td>Serum alkaline phosphatase a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment period</td>
<td>4.88</td>
<td>5.03</td>
<td>5.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment period</td>
<td>4.73</td>
<td>5.47</td>
<td>5.21</td>
<td>0.25</td>
<td>11.8</td>
</tr>
<tr>
<td>Post-treatment period</td>
<td>4.66</td>
<td>4.86</td>
<td>4.72</td>
<td>0.26</td>
<td>13.5</td>
</tr>
</tbody>
</table>

a SE = Standard error of a treatment mean.
b C.V. = Coefficient of variation.
c Value at time skin biopsies were taken and when feeding of experimental diets was initiated.
d Sigma units/milliliter.

of blood has been observed previously when high levels of supplemental zinc were fed (9, 14). However, in most previous studies, only one form of zinc (usually zinc oxide) has been used. In this study the highly soluble form of zinc (zinc sulfate) had no greater influence on blood zinc level than the relatively insoluble form (zinc oxide).

Serum alkaline phosphatase values were not materially affected by either form of the supplemental zinc (Table 1). These data are in agreement with results with cows which indicate that serum alkaline phosphatase was not affected by feeding high levels of supplemental zinc (9). Serum alkaline phosphatase values are much lower in zinc-deficient calves (12).

Rate of wound healing was not appreciably affected by either form of the supplemental zinc (Tables 2 and 3). However, three measures of treatment effect differed significantly at the 5% probability level (Tables 2 and 3). These were: a) width for the third week measurement; b) length of the unhealed diagonal for the fourth week; and c) area of the wound for the fourth week. None of the treatment effects for the first two or the last two weeks was significant at the 5% level of probability. While it is possible that some small treatment effects may have occurred, it appears likely that the differences observed were largely due to chance.

In previous work with calves, rate of wound healing was reduced in zinc-deficient calves compared to ad libitum-fed controls (11). Also, restricting the level of intake of calves fed the control diet to a level somewhat below that of the zinc-deficient animals reduced skin regeneration (11). However, the type of wound healing was normal in the calves fed the control diet in restricted amounts, but quite abnormal in the deficient animals (11). In these experiments treatment effects were quite large from two weeks after surgery until new skin had completely covered the area (11). In the human studies the treatment effect was much larger after two weeks following surgery than before two weeks after surgery.2

The data herein reported indicate that adding supplemental zinc to a practical-type diet had little or no effect on wound healing in calves. This is in contrast to studies with humans in which supplemental zinc sulfate greatly increased rate of healing.2 However, in addition to the species and sex differences there were also differences in the type of wounds. The calf wounds were only skin deep and so small as to have no apparent effect on animal health and comfort. In the human studies, wounds were relatively much larger and involved loss of tissue down to fascia, including fat as well as skin.2 Thus, it is not definitely known whether the type of wound has an effect on the response...
TABLE 2

Effects of diets containing supplemental zinc oxide and zinc sulfate on rate of skin regeneration in Holstein heifers as determined from measurements of the unhealed area

<table>
<thead>
<tr>
<th>Length</th>
<th>Width</th>
<th>Area</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Week 0</th>
<th>Control</th>
<th>Zinc oxide</th>
<th>Zinc sulfate</th>
<th>Treatments combined</th>
<th>Avg</th>
<th>SE*</th>
<th>F ratio (treatment effect)</th>
<th>LSDb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>57.6</td>
<td>56.7</td>
<td>57.6</td>
<td>57.6</td>
<td>57.6</td>
<td>0.64</td>
<td>0.58</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>52.7</td>
<td>53.7</td>
<td>52.9</td>
<td>53.4</td>
<td>52.7</td>
<td>0.64</td>
<td>1.19</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>34.3</td>
<td>34.5</td>
<td>35.4</td>
<td>34.8</td>
<td>34.7</td>
<td>2.63</td>
<td>0.58</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>23.6</td>
<td>23.2</td>
<td>25.4</td>
<td>24.1</td>
<td>24.1</td>
<td>1.51</td>
<td>1.78</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>16.5</td>
<td>17.6</td>
<td>19.9</td>
<td>17.5</td>
<td>18.6</td>
<td>0.87</td>
<td>0.86</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>12.7</td>
<td>13.3</td>
<td>14.8</td>
<td>14.6</td>
<td>13.6</td>
<td>0.63</td>
<td>1.05</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>8.0</td>
<td>8.8</td>
<td>10.1</td>
<td>7.7</td>
<td>0.67</td>
<td>0.97</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>57.6</td>
<td>53.4</td>
<td>52.7</td>
<td>53.4</td>
<td>52.7</td>
<td>0.64</td>
<td>1.19</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>34.3</td>
<td>34.5</td>
<td>35.4</td>
<td>34.8</td>
<td>34.7</td>
<td>2.63</td>
<td>0.58</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>23.6</td>
<td>23.2</td>
<td>25.4</td>
<td>24.1</td>
<td>24.1</td>
<td>1.51</td>
<td>1.78</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>16.5</td>
<td>17.6</td>
<td>19.9</td>
<td>17.5</td>
<td>18.6</td>
<td>0.87</td>
<td>0.86</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>12.7</td>
<td>13.3</td>
<td>14.8</td>
<td>14.6</td>
<td>13.6</td>
<td>0.63</td>
<td>1.05</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>8.0</td>
<td>8.8</td>
<td>10.1</td>
<td>7.7</td>
<td>0.67</td>
<td>0.97</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*SE = Standard error of a treatment mean.
*b LSD = Least-significant difference at the 5% probability level, based on variances observed in this experiment. Calculated using the t value for 60 d.f. for error and 24 observations per treatment. The LSD's are expressed in number of days required to be significant, assuming the skin growth rate shown for each week.

The measurements are the length and width of the diagonals and the area of the portion over which new skin had not grown.

* Indicates difference was significant at the 5% probability level.

of animals to supplemental zinc. While no dietary restrictions were imposed on the humans, the average zinc content of the diet was not defined.7

Because of its importance in medicine, the relationship of nutrition to wound healing has been studied in many experiments (3, 4). Predominantly, these studies have been with rats or other small laboratory animals. Apparently, the most frequently used procedure has been that of determining adhesion strength of a sutured wound after specified time intervals. It was reasoned that for cattle a procedure involving measurement of skin regeneration as performed in this study would have a number of advantages. Very useful results were obtained with such a study, as demonstrated in a previous publication (11). However, at that time the method was not critically evaluated.

Data in Table 3 show the means squares attributed to the three diets, the duplication on sides of the animals, the influence of two independent observers, the interactions, and the residual variance.

Two relatively untrained observers independently obtained measurements that were in good agreement. On some occasions, the presence of scabs obscured the exact demarcation line between healed and unhealed areas. In such instances variability between observers tended to be larger.

Rate of skin regeneration was not uniform throughout the healing period. The rate was moderately rapid the first week, accelerated greatly the second week, declined through the fifth week, then leveled off to a rate comparable to that of the first week. A similar pattern was observed previously (11). Data indicate that
the growth of the skin proceeded in a linear pattern all around the wound. Differences between the diagonals indicated as width and length apparently are a reflection of the sharpness of the angles at the points. Standard errors of treatment means for the linear measurements did not increase materially throughout the 6-wk period, suggesting that variability among animals within treatment did not increase greatly with time. Thus, random errors not associated with animal-by-time interactions were predominant sources of variation.

Variance components were broken down and that attributed to each source expressed as a percentage of the total (Table 4). The range in the percentages of the variance accounted for by the residual component was from 81.7 to 100 for the different measurements and times studied (Table 4). These data indicate that the two observers and two sides had relatively little effect in reducing variances. Likewise, interactions with observers and sides were unimportant. All sources of variation except the residual (animal within treatment, within side, and within observer) were small. Thus, the most effective way to increase treatment precision would be to use larger numbers of animals.

Since the residual effect (Table 4) is the only component making any sizable contribution to the variance, it seemed important to determine if some of the other variables, for which data were available, were affecting rate of healing. Relationship of initial weight, initial age, and rate of gain with rate of healing was studied by calculating the simple correlations of these variables with the various measurements of the unhealed wound areas (Table 5). Ranges of the independent variables were as follows: initial weight, 108-170 kg; initial age, 128-222 days; and rate of gain, 0.68-1.16 kg per day. All of the correlations were small and tended to average near zero. Thus, within the ranges studied, age, weight, and rate of gain did not have an important effect on rate of skin regeneration. Accordingly, it is not important to select animals for uniformity in these characteristics for wound healing studies. The response of individual animals was somewhat variable, but the cause was not discovered.

The least significant differences (Table 2) needed to obtain significance between treatment means were calculated on the basis of 60 d.f. for error and 24 observations per treatment. Assuming the same variability, if the data were based on single observations per calf resulting
TABLE 4
Per cent of total variance accounted for by each variance component. A completely random model is assumed

\[
\begin{array}{cccccccc}
\text{Treatment} & \text{Side} & \text{T×S} & \text{Observer} & \text{T×O} & \text{S×O} & \text{T×S×O} & \text{Residual}^b \\
(T) & (S) & & & & & & \\
\hline
\text{Length}^a & & & & & & & \\
\text{Week 1} & 0.0 & 0.1 & 0.0 & 0.0 & 0.0 & 0.0 & 99.9 \\
\text{Week 2} & 0.0 & 0.0 & 0.0 & 1.3 & 0.0 & 0.0 & 98.7 \\
\text{Week 3} & 5.2 & 0.0 & 0.0 & 0.0 & 0.0 & 0.0 & 94.8 \\
\text{Week 4} & 11.7 & 0.4 & 0.0 & 0.0 & 0.0 & 0.0 & 97.4 \\
\text{Week 5} & 0.4 & 5.3 & 0.0 & 0.0 & 0.0 & 0.0 & 94.3 \\
\text{Week 6} & 0.0 & 7.1 & 5.3 & 0.0 & 0.0 & 0.0 & 97.6 \\
\hline
\text{Area} & & & & & & & \\
\text{Week 1} & 1.3 & 0.6 & 0.0 & 2.8 & 0.0 & 0.0 & 95.3 \\
\text{Week 2} & 0.0 & 0.0 & 0.0 & 0.0 & 0.0 & 0.0 & 100.0 \\
\text{Week 3} & 6.7 & 0.0 & 0.0 & 0.0 & 0.0 & 0.0 & 93.3 \\
\text{Week 4} & 9.8 & 0.0 & 0.0 & 0.0 & 0.0 & 0.0 & 90.2 \\
\text{Week 5} & 3.0 & 1.4 & 0.0 & 0.0 & 0.0 & 0.0 & 94.8 \\
\text{Week 6} & 3.5 & 6.9 & 5.8 & 0.0 & 0.0 & 0.0 & 89.6 \\
\end{array}
\]

\text{Residual} is the animal within treatment, within side, and within observer component.

\text{Width data are omitted to conserve space. For Weeks 1-6, percentages of the total variance in width data attributed to residual were 91.6, 96.5, 81.7, 85.9, 92.6, and 84.3.}

\text{All negative values are considered to be 0, as no component can contribute a negative value.}

in 15 d. f. for error and six observations per treatment, the difference in rate of healing required for significance at the 5% probability would have been a little more than twice the values shown (Table 2). In all instances the area measurement was slightly more sensitive than the diagonals, which generally were about the same. This is not surprising, as the area data are calculated from the diagonals.

Measured in terms of numbers of days' difference, due to treatments in healing progress, which would be required for treatment effects to be significant, the method appears to be quite sensitive (Table 2). For most of the measures, a difference in healing rate of from one to four days would have been significant at the 5% probability level (Table 2). When considered in light of the fact that a difference in treatment effects of 2.5 wk was observed in a previous study, due to a zinc deficiency or restricted feed intake (11), the sensitivity of the method appears to be quite satisfactory.

It is becoming widely recognized that optimum nutrition must include much more than satisfactory weight gain, milk production for short periods, and the other usual measures of animal performance. However, whether rations adequate for normal performance are also satisfactory for other parameters of nutritional adequacy has been studied relatively little in cattle. One of the parameters is wound healing. Results reported in this paper, along with previous work (11), show that rate of skin regeneration can be used as an effective way of studying wound healing in cattle. The procedure is relatively simple and easily applied, with the main special skill needed being that of the surgeon making the wounds.

TABLE 5
Simple correlations of weight gains, initial age, and initial weight with measurements of the unhealed wound areas

\[
\begin{array}{ccc}
\text{Weight} & \text{Initial} & \text{Initial} \\
\text{gain} & \text{age} & \text{weight} \\
\hline
\text{Length}^a & & \\
\text{Week 1} & -0.19 & -0.27 & -0.09 \\
\text{Week 2} & -0.10 & 0.19 & -0.22 \\
\text{Week 3} & 0.11 & 0.36 & 0.25 \\
\text{Week 4} & 0.07 & -0.03 & -0.03 \\
\text{Week 5} & 0.33 & 0.21 & 0.31 \\
\text{Week 6} & 0.11 & 0.10 & 0.23 \\
\text{Avg} & 0.06 & 0.08 & 0.11 \\
\hline
\text{Area} & & \\
\text{Week 1} & -0.14 & -0.13 & 0.05 \\
\text{Week 2} & 0.04 & 0.30 & 0.23 \\
\text{Week 3} & 0.14 & 0.38 & 0.19 \\
\text{Week 4} & 0.15 & 0.07 & 0.01 \\
\text{Week 5} & 0.22 & 0.06 & 0.10 \\
\text{Week 6} & -0.18 & -0.16 & -0.10 \\
\text{Avg} & 0.04 & 0.09 & 0.08 \\
\end{array}
\]

\text{Width data are omitted to conserve space.}

\text{Values are the simple correlations between an independent variable and a measure of unhealed area. Thus, a negative correlation indicates a positive relationship between the variable and rate of skin regeneration.}

J. DAIRY SCIENCE VOL. 50, NO. 5
Acknowledgments

The authors thank James G. Fortson and J. L. Carmon for assistance in statistical analyses and interpretations; H. F. Perkins for assistance in the zinc analyses; and J. V. Mason for alkaline phosphatase determinations. Appreciation is extended to Dawes Laboratories, Chicago, Illinois, for vitamins; to American Cyanamid Company, Princeton, New Jersey, for chlortetracycline; and to the Smith-Douglas Company, Norfolk, Virginia, for defluorinated rock phosphate.

References