VALUE OF IMMUNOHISTOCHEMISTRY IN BONE MARROW DIAGNOSIS IN ANEMIC RATS

Farah, K. M.

Department of Clinical Pathology, Faculty of Veterinary Medicine, Benha University

ABSTRACT

The present study was performed to evaluate usefulness of immunohistochemistry on routinely processed bone marrow specimens for diagnostic purposes in anemic rats. Rats were divided into three groups in a completely randomized design. Group A was the control. Group B & C received zinc chloride (20mg/kg body weight) for 2 and 4 months respectively. Significantly low percentages of LN-1 (monoclonal antibody for RBC precursor cells) positive bone marrow cells were found in zinc chloride administered groups compared to control one. Significant decrease was found in WBCs, RBCs counts, Hb concentration, and PCV value in peripheral blood of zinc chloride treated groups with slight anisocytosis. Biochemical parameters showed significant decrease in serum copper, ceruloplasmin and iron, while total iron binding capacity (TIBC) showed significant increase. It was concluded that overdoses of zinc therapy, which is often taken for health enhancement, resulted in normocytic hypochromic anemia with leukopenia. Bone marrow examination using immunohistochemistry facilitate the recognition of hypoplasia and dyserythropoiesis induced by high dose zinc administration.

INTRODUCTION

Bone marrow biopsy has a difficulty in diagnosis due to morphological differences between the different types of cells and their stages of maturation (Hall et al 1987). Also hematopoietic tissues and stromal cells have a contrasting texture, so that need a high quality sections (Linder et al 1986). Immunohistochemistry play a role in bone marrow diagnosis and differentiation between variety of lymphoproliferative, myeloproliferative and metastasis disorders (P. Van et al 1989). A panel of antibodies can be used to stain different cell line, LN-1 used for erythrocytes, LN-2 and LCA for leukocytes, MB-2 for lymphocytes, and CD-68 for macrophage and mast cells.
Copper is known as an essential trace element and is a component of metalo-enzymes and ceruloplasmin. Acquired copper deficiency due to zinc chloride over doses is characterized by hematological and bone marrow changes including bone marrow depression, anemia and neutropenia (Fiske et al 1994).

The aim of the present work was to immunologically characterize the erythropoietic cell line in normal and anemic rats and to evaluate the usefulness of immunohistochemistry in bone marrow diagnosis.

**MATERIAL AND METHODS**

**Animals:**

Thirty male rats (80-110 g body weight, age from 1.5-2 months) were used in this study. The animals were divided into 3 groups (10 animals per group). Group (A) was the control; group (B) was orally administrated zinc chloride 20 mg/kg body weight, dissolved in distilled water daily for 2 months. Group (C) was orally administrated zinc chloride 20 mg/kg body weight, dissolved in distilled water daily for 4 months.

**Chemicals:**

Zinc chloride, Tris Buffer, and skim milk were obtained from Sigma Chemical, USA. LN-1 (immunoglobulin for RBC cells) and anti-mouse immunoglobulin (horse anti-mouse IgG) were obtained from Dako Corporation, USA.

**Blood tests:**

Blood samples were collected on EDTA. The red blood cell count, Hb concentration, and PCV percentage were performed according to Duncan, et al. (1994). Serum samples were also prepared for performing blood chemistry tests. Kits were obtained from Quimica Clinica Aplicada S.A. Company. Iron and Cu, were measured according to Kang et al (1992), TIBC according to Gottschalk et al (2000), Ceruloplasmin according to Out et al (1987)
Bone marrow:

- Rats were killed by cervical dislocation 24 hours after zinc chloride administration. Both femurs were dissected out from each animal. The contents of each femur were removed out.
- Bone marrow samples were fixed in 10% neutral formalin and decalcified in neutral EDTA for 24-72 hours
- Dehydration in alcohol (75-100%) and then embedded in paraffin
- Sections were stained with H&E and immunohistochemical stains. Bone marrow smears were also prepared, fixed in methanol for 10 minutes and stained with Geimsa stain after drying.

Immunohistochemical staining:

- It was performed according to Summerfeild et al (1992)
- All paraffin sections were deparafinized in xyline and dehydrated in alcohol
- Then incubation for 30 minutes in methanol with 0.5 % H₂O₂ (to block endogenous peroxidase) was made.
- Sections were washed in Tris Buffer saline (TBS) for 5 minutes.
- Block in 5 % skim milk for 10 minutes
- Incubation with first antibody (LN-1) for overnight at 4 °C
- Rinsed in TBS for 5 minutes
- Incubate for 60 minutes with anti-mouse immunoglobin (horse anti-mouse IgG) at room temperature.
- Incubate with ABC (avidin-biotin complex) kit for 50 minutes
- Rinse in TBS three times for 5 minutes
- Incubate with DAB (3,3-diamino benzidene tetrahydrochloride) in tris Hcl for 5-10 minutes.
- Rinse in phosphate buffer saline (PBS) three times for 5 minutes.
- Wash with distilled water
- Counter stain with H&E for few seconds.
- Dehydrate in alcohol and xyline
- Mount and cover with Canada balsam.
Examination of bone marrow film

One thousand nucleated cells were counted and the percentage of positive cells was analyzed (Motomura et al. 2001).

Statistical analysis

Data are expressed as the mean ± SE. Differences between means were analyzed by students't test according to Snedecor and Cochran (1994)

RESULTS

Bone marrow:

Figure 1 shows H&E, Geimsa and immunohistochemical staining of LN-1 positive cells from the bone marrow. LN-1 stained the immature erythroid cells. Positive cells showed an intracytoplasmic intense dot-like brown reaction.

The values of LN-1 positive cells in zinc chloride-administered groups were significantly low than that of normal animals as shown in table 1 and figures 2 & 3.

Biochemical changes:

Table 2 shows the changes in the biochemical parameters. Significant decrease was found in serum copper, ceruloplasmin and iron, while total iron binding capacity (TIBC) showed significant increase in 2 and 4 months zinc chloride-treated group respectively compared to the control.

Hematological changes:

Significant decrease was found in WBCs, RBCs counts, Hb concentration, and PCV value in zinc chloride-treated groups as shown in table (2). Red cell indices showed normocytic hypochromic anemia. The peripheral blood smears showed slight anisocytosis.
Fig 1: Bone marrow specimens stained with H&E, Geimsa, and Immunohistochemical (LN-1) stains.

![Bone marrow specimens stained with H&E, Geimsa, and Immunohistochemical (LN-1) stains.]

Fig 2: Immunohistochemical staining of LN-1 positive cells in bone marrow from normal (A), after 2 months (B), and after 4 months (C) of chloride administration.

![Immunohistochemical staining of LN-1 positive cells in bone marrow from normal (A), after 2 months (B), and after 4 months (C) of chloride administration.]

Table 1: LN-1- positive cells in bone marrow smears from normal, after 2 and 4 months of zinc chloride administration.

![Table 1: LN-1- positive cells in bone marrow smears from normal, after 2 and 4 months of zinc chloride administration.]
<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>After 2 months</th>
<th>After 4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN-1</td>
<td>16.1 ± 0.48</td>
<td>10.85 ± 0.56***</td>
<td>9.2 ± 0.46***</td>
</tr>
</tbody>
</table>

Values are the mean ± S.E., n = 10. ***P<0.001 vs. control group.

**Fig 3:** LN-1- positive cells in bone marrow smears from normal, after 2 and 4 months of zinc chloride administration.
Table 2: Hematological and biochemical changes in normal and zinc chloride administered groups

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>After 2 months</th>
<th>After 4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs x 10^3</td>
<td>8.13 ± 0.30</td>
<td>7.15 ± 0.23*</td>
<td>6.1 ± 0.24**</td>
</tr>
<tr>
<td>RBCs x 10^6</td>
<td>5.23 ± 0.32</td>
<td>4.16 ± 0.29***</td>
<td>3.93 ± 0.27***</td>
</tr>
<tr>
<td>Hb g/dl</td>
<td>11.92 ± 0.36</td>
<td>9.39 ± 1.07**</td>
<td>9.03 ± 0.67**</td>
</tr>
<tr>
<td>PCV %</td>
<td>42.25 ± 0.81</td>
<td>34.00 ± 0.69***</td>
<td>33.23 ± 0.73***</td>
</tr>
<tr>
<td>MCV fl</td>
<td>8.07 ± 0.49</td>
<td>8.17 ± 0.71</td>
<td>8.32 ± 0.65</td>
</tr>
<tr>
<td>MCH pg</td>
<td>20.44 ± 1.21</td>
<td>22.57 ± 2.31</td>
<td>24.87 ± 2.85</td>
</tr>
<tr>
<td>MCHC %</td>
<td>28.20 ± 2.10</td>
<td>27.61 ± 3.24</td>
<td>27.01 ± 4.2*</td>
</tr>
<tr>
<td>Copper µmol/L</td>
<td>15.79 ± 0.46</td>
<td>9.09 ± 0.63***</td>
<td>6.27 ± 0.57***</td>
</tr>
<tr>
<td>Ceruloplasmin mg/L</td>
<td>150.5 ± 3.85</td>
<td>102.00 ± 5.00***</td>
<td>90.10 ± 3.71***</td>
</tr>
<tr>
<td>Iron µmol/L</td>
<td>24.50 ± 1.23</td>
<td>16.90 ± 0.76***</td>
<td>10.10 ± 0.70***</td>
</tr>
<tr>
<td>TIBC µmol/L</td>
<td>42.60 ± 1.18</td>
<td>46.90 ± 0.82*</td>
<td>53.90 ± 1.76***</td>
</tr>
</tbody>
</table>

Values are the mean ± S.E., n = 10. *P<0.05 vs. control group.

DISCUSSION

Copper deficiency characteristically causes anemia and neutropenia (Patterson et al 1985, Simon et al 1988). Acquired copper deficiency has been reported with long term excess zinc intake (Al-Rashid and Spangler 1971, Dunlap et al 1974). Zinc is supplemented because it is important in the treatment of intestinal mal-absorption, sickle cell anemia and variety of other reasons (Brewer et al 1983). However, it must be stressed that high doses of oral zinc can result in copper deficiency. As copper and zinc enter the intestinal mucosal lining cells, they are bound by metallothionein, which is an intracellular ligand (Webb and Cain 1982). Its synthesis is influenced directly by dietary content and serum zinc level. Excessive intestinal zinc increases the synthesis of metallothionein by the epithelial cells, which in turn results in increased intracellular binding and stagnation, thus limiting further zinc absorption. The zinc-laden intestinal
cells are then sloughed and lost in the feces. Copper has been shown to have a higher affinity for metallothionein than zinc. The bound zinc is displaced by copper because of its higher binding affinity, resulting in reduced delivery of copper to the blood and increased fecal excretion of copper (Ringenberg et al 188). Copper is necessary for incorporation of iron into the heme molecule (Day et al 1988). Zinc-induced copper deficiency impairs the transport of iron by decreasing level of ceruloplasmin ferroxide. Also, the level of copper dependent plasma growth factors and intracellular copper-dependent enzymes (such as cytochrome oxidase, a mitochondrial enzyme necessary for mitochondrial iron utilization and heme synthesis) are depressed (Williams et al 1976, Williams 1983).

In the present study significant decrease in WBCs & RBCs counts, Hb concentration, and PCV value in zinc-treated groups after 2 and 4 months of treatment. Slight anisocytosis was found in the peripheral blood smears. Red cell indices showed normocytic hypochromic anemia. These finding represents a zinc-induced copper deficiency anemia and leukopenia.

Biochemical parameters showed significant decrease in serum copper, ceruloplasmin and iron levels; whereas total iron binding capacity showed significant increase. These results agree with Tamura et al (1994).

Bone marrow examination of zinc-administered groups showed significant decrease of LN-1 positive erythroid cells after 2 and 4 months of administration. No significant difference of LN-1 positive erythroid cells between 2 and 4 months treated groups. This may be due to myelodysplastic syndrome (maturation defect and ineffective hematopoiesis) induced by copper deficiency (Summerfeild et al 1992).

The results of this study show that immunohistochemistry is a reliable on routinely processed bone marrow specimens as it gives a good morphology for sections and positive cells. Immunohistochemistry can contribute in anemia diagnosis since it allows for quick recognition of the proportion of the different cell lines in the bone marrow and evaluation of qualitative changes in certain cell types after it is marked with specific antibody such as LN-1 for erythroid cell line (P Van Der Valk et al 1989, Orazi et al 1994).
In summary, overdoses of zinc therapy, which is often taken for health enhancement, resulted in anemia with leukopenia. Bone marrow examination using immunohistochemistry facilitate the recognition of hypoplasia and dyserythropoiesis induced by high dose zinc administration

REFERENCES


Benha Ras Sedr 25-28 January 2007


