Iron Status and Stores Decline with Age in Lewis Rats

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ABSTRACT In the context of a larger study examining the interaction of vitamin A (VA) status and age on immune function, we examined age-related changes in hematologic and iron status variables in male Lewis rats. Animals were fed a nutritionally adequate purified diet containing either 0.35 (marginal), 4.0 (control) or 50 (supplemented) mg retinol equivalents (as retinyl palmitate) per kg of diet from the time of weaning until killing at 8–10 (middle-aged) or 20–22 (old) mo of age. Neither VA nor VA and age interaction effects were significant for most iron variables examined. After controlling for body weight, old rats had significantly lower hemoglobin, hematocrit and plasma iron than middle-aged rats. This decrease in hematologic and transport iron variables was not accompanied by a shift of iron into other storage compartments. Old rats also had significantly lower total iron content and iron concentration in liver, spleen and bone marrow. Hemosiderin iron in marrow smears correlated significantly (r = 0.43–0.76, P < 0.05) with chemical estimates of iron in storage, transport and functional pools. Old rats also tended to have less stained iron in femur marrow smears. Thus, body iron in functional, transport and storage compartments, namely the liver, spleen and bone marrow, were significantly lower in old than in middle-aged rats. Although iron stores and status are usually considered to increase with advancing age, our data show a consistent pattern of lower hematologic and storage iron variables in old than in middle-aged Lewis rats. Future research is indicated to understand the biology and functional consequences of the observed age-associated decline in body iron.


KEY WORDS: • aging • iron status • iron stores • bone marrow smears • rats

Aging is associated with changes in metabolism and status of various nutrients. There is some evidence that hematologic variables such as hemoglobin and hematocrit may decline with aging in humans and in animal models (Chen and Cook-Newell 1989, Inelmen et al. 1994, Lipschitz 1991, Preziosi et al. 1994, Salive et al. 1992, Williams et al. 1986) while others have reported no age-related changes (Garry et al. 1983, Zauber and Zauber 1987). There is also evidence suggesting that serum ferritin, which is usually indicative of total body iron stores (Walters et al. 1973), increases with aging (Cals et al. 1994, Cook et al. 1976, Milman et al. 1986). However, these findings must be interpreted with caution because low hemoglobin and hematocrit may be secondary to age-associated pathology, acute or chronic infections or inflammation, other nutrient deficiencies and use of prescription or over-the-counter drugs (Lipschitz et al. 1984, Lipschitz 1987, Murphy and Hutchinson 1994). Furthermore, normal to high serum ferritin concentrations in older adults may not accurately reflect iron status. Ferritin is an acute phase reactant protein which increases in response to underlying infection, inflammation or disease regardless of the individual’s iron status (Guyatt et al. 1990, Hillman and Finch 1985, Touitou et al. 1985). Therefore normal-to-elevated serum ferritin levels in an older population, where overt and subclinical infections may be more prevalent, can mask iron deficiency (Ahluwalia et al. 1995, Joosten et al. 1993).

Evaluation of bone marrow hemosiderin iron is usually regarded as the reference standard for assessment of iron stores (Hillman and Finch 1985, Holyoake et al. 1993). It is considered as the most sensitive and specific indicator of body iron stores and unlike other conventional laboratory measures of iron status, it is not confounded by infection or inflammation (Hillman and Finch 1985). This technique, however, is costly, invasive, painful and impractical for routine examinations and studies. Thus, studies of changes in bone marrow iron with aging in humans and animals are limited (Benzie 1963, Takeda et al. 1996). Liver and spleen are the other two chief sites for iron storage (Hillman and Finch 1985). Examination of iron stores in liver and spleen of mice and rats have shown, for the most part, that older animals have significantly higher iron in these tissues (Cook and Yu 1998, Massie et al. 1983, Sohal et al. 1999, Takeda et al. 1996). However, a significant aging effect in most of these studies has usually been concluded over a wide age range, including data from young growing animals. Furthermore, literature on this topic is difficult to assimilate due to differences in the definitions used for aging and old age, species and strain differences, and because most studies did not control for potentially confounding variables such as body weight.

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We were, therefore, interested in a comprehensive evaluation of iron status, involving simultaneous examination of hematological and storage iron variables including bone marrow iron stores, with aging from middle age to old age in an animal model. We had the opportunity to examine this issue in the context of another study designed to evaluate the interaction of vitamin A (VA) status and age on immune function in Lewis rats (Dawson et al. 1999, Dawson and Ross 1999). Specifically, we examined hematologic and transport indices as well as iron content in primary organs for iron storage, namely liver, spleen and bone marrow, in middle-aged as compared to old Lewis rats.

**MATERIALS AND METHODS**

**Animals, diets and experimental design.** The animal protocol used in these studies was approved by the Animal Use and Care Committee of The Pennsylvania State University. The details of animals, diets and experimental design have been previously described by Dawson et al. (1999). Briefly, the parent study was a 3 x 3 two-factorial design to examine the interaction of age and VA status on immune function. From the time of weaning, male Lewis rats were fed a nutritionally adequate diet, the AIN-93M rodent diet (Reeves et al. 1993), obtained commercially (Dyets, Bethlehem, PA), with a chemically estimated iron content of 40.3 mg/kg diet, modified to contain 0.35, 4.0 or 50.0 mg RE (in the form of retinyl palmitate)/kg diet. These levels of dietary VA were designated marginal, control and supplemented, respectively. Rats were fed these diets until they were 2–3, 8–10 or 20–22 mo of age. These ages were designated young, middle-aged and old, respectively. Because the purpose of the current study was to examine changes in iron status with aging, the young group, which included rapidly growing animals, was not included in the present analyses. Therefore, to examine iron status with aging in adult animals, the current study was limited to a 2 x 3 two-factorial design; age had two levels (middle-aged and old) and dietary VA had three levels (marginal, control and supplemented).

**Bone marrow examination for hemosiderin iron.** Bone marrow from the proximal femur from the other leg was used to prepare smears on glass slides (3–4 per rat) and stained for iron using Gomori’s method (Armed Forces Institute of Pathology, 1992). Smears were examined by light microscopy and graded by three observers (a pathologist, G. H. and two experienced observers, N. A. and M. G.). Prior to actual grading of slides, the entire spectrum of slides from the current study was reviewed in a couple of batches to define grades of iron from “absent” to “increased.” At each marrow smear examination session, the slides defining various grades of iron were first reviewed to serve as internal controls. Smears were then graded in seven batches; each batch included numbered slides from rats of each of the six subgroups in the 2 x 3 factorial design. However, the observers were unaware of the age or dietary VA assignment for rat numbers. Smears were graded semiquantitatively on a scale of 0 to 3; 0 = absent, 1 = decreased, 2 = adequate, and 3 = increased iron deposits, based on the intensity of iron stain after examination under low (X100) and high (X200-X400) power as necessary. Zero iron was defined as no identifiable iron staining after examination under high power (X400). The interobserver concordance on smear grading was 93%. For the other 7% of cases, slides were immediately reexamined until concordance was reached upon discussion and deliberation as needed.

**Statistical analysis.** Statistical analyses were carried out using PC-SAS (SAS Institute, Cary, NC). Distributions for continuous variables were examined and found to be consistent with normality; therefore raw data were used for analyses. Given the variations in dietary VA intake among study animals, the main effects of dietary VA and interaction effects of dietary VA and age on various iron variables examined were first evaluated using ANOVA. Neither dietary VA main effect nor the interaction of age and dietary VA was significant for most of the iron-status variables examined (P > 0.10). Therefore, animals in various dietary VA levels were pooled within each age to examine the main effect of age. For bone marrow and spleen iron-related variables, age and dietary VA interaction was significant; therefore dietary VA was used as a covariate to examine the main effect of age for these variables. Because body weight correlated significantly with most iron-status variables examined, it was used as a covariate, as in the approach of Roedenburg et al. (1996), in the ANOVA model to examine the main effect of age. Spearman rank correlation coefficients were obtained between femur marrow smear iron grade and quantitative variables of iron status. A P-value < 0.05 was considered significant. Values are means ± SEM.

**RESULTS**

Hematological and iron transport measurements of iron status were significantly lower in old than in middle-aged rats (Table 1, P = 0.0001); hemoglobin, hematocrit and plasma iron in old rats were 79, 87 and 54%, respectively, of the levels in middle-aged rats.

Total iron content of liver, spleen and femur marrow in old rats was also significantly lower; levels in old rats were 41, 65 and 62%, respectively, of the levels in middle-aged rats (Table 2, P < 0.05). When tissue iron in these primary sites of iron storage were summed, old rats had less than half (46%) the iron stores of middle-aged rats (adjusted mean ± SEM: 3854 ± 216 and 1781 ± 245 for middle-aged and old rats, respectively; P = 0.0001). A similar trend of age-associated decline in iron concentration was also seen; iron concentration in liver, spleen and bone marrow in old rats was about 47, 34 and 41% of middle-aged levels, respectively (Table 2, P < 0.001).

Bone marrow hemosiderin iron was examined in smear obtained from femur marrow. Average iron grade in femur marrow smears after controlling for body weight and dietary VA was 2.14 (±0.14) and 0.20 (±0.15) for middle-aged and old Lewis rats, respectively.

**TABLE 1**

<table>
<thead>
<tr>
<th>Age</th>
<th>n</th>
<th>Hemoglobin, g/L</th>
<th>Hematocrit</th>
<th>Plasma iron, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle-aged</td>
<td>48</td>
<td>186.1 ± 3.5</td>
<td>53.3 ± 0.008</td>
<td>42.9 ± 1.8</td>
</tr>
<tr>
<td>Old</td>
<td>43</td>
<td>147.8 ± 3.8</td>
<td>46.4 ± 0.009</td>
<td>23.2 ± 2.0</td>
</tr>
</tbody>
</table>

*Abbreviation used: VA, vitamin A.

*Values are adjusted means ± SEM.*
old rats, respectively. The distributions of femur marrow smear iron grade in animals fed control diet, i.e., a nutritionally complete diet which also provided adequate dietary VA (Dawson et al. 1999), are shown for middle-aged and old rats in Figure 1. Most middle-aged rats (93%) had normal hemosiderin iron (grade = 2) in their femur marrow smears as compared to only 15.3% old rats. A greater proportion of old rats tended to have reduced or absent iron stores (grades 0 and 1, respectively) in their marrow smears as compared to middle-aged animals (Fig. 1). However this trend could not be evaluated statistically because of small number of animals in various levels of marrow smear iron grade. A noteworthy finding was that marrow smear iron grade correlated significantly with chemical estimates of nonheme iron stores in bone marrow, liver and spleen and with iron transport and hematologic variables (Table 3, P < 0.05).

DISCUSSION

The examination of iron status with aging has gained further interest in recent years with several reports suggesting that aging is associated with increased lipid peroxidation and oxidative damage to tissues (Cook and Yu 1998, Mecocci et al. 1999, Sohal 1994, Wei et al. 1996), and because iron is a catalyst for lipid peroxidation (McCord 1996). It is difficult to gauge iron status, particularly iron stores, based on traditional laboratory tests which are often confounded by the presence of infection, inflammation or disease in older adults (Ahluwalia et al. 1995, Hillman and Finch 1985, Holyoke et al. 1993). Several investigations have, therefore, focused on examination of tissue iron stores with aging in various animal models. The issue of iron homeostasis from middle- to old-age in a controlled setting and in a comprehensive manner, however, is underexamined. Therefore, we conducted a comprehensive evaluation of iron status including hematologic and transport iron variables as well as iron content in primary sites of iron storage, namely liver, spleen and bone marrow, in old as compared to middle-aged Lewis rats.

This study was conducted in the context of the parent study examining the interaction of age and dietary VA on immune function. Because of the increasing evidence supporting interactions between VA status and iron status (Bloem et al. 1990, Roodenburg et al. 1996, Rosales et al. 1999), we first examined the effects of dietary VA, and the interaction of dietary VA and age on iron-status variables. For most iron-status variables examined, these effects were not significant; this might be because, in the current study, VA status varied from marginal to supplemented but excluded VA deficiency and toxicity. Therefore, data were pooled across dietary VA to examine changes in iron-status variables with aging; dietary VA was used as a covariate when indicated, as in the case of spleen iron and bone marrow iron-related variables. Because the two-age groups had significantly different body weight and body weight was correlated with several of the iron status variables examined, we also used body weight as a covariate in these analyses, as in the approach of Roodenburg et al. (1996).3 Food-intake data were available through 16 mo of age; during this time, food intake was similar regardless of VA intake or age, and body weight was not related to food intake (Li, N.-Q. and Ross, A.C., unpublished observations and Dawson et al. 1999). After controlling for body weight, most of the iron status variables examined were significantly lower in old than in middle-aged rats.

Age-related decline in hematological variables has been the subject of extensive investigation. Our results indicate that in this animal model, aging was associated with a significant decline in hemoglobin (by 38.3 g/L) and hematocrit (by 0.07) after controlling for body weight. These findings are consistent with those from several epidemiologic studies, indicating lower hemoglobin levels with advancing age (Chen and Cook-Newell 1989, Inelmen et al. 1994, Lipschitz 1991, Preziosi et al. 1994, Salive et al. 1992). Other investigators (Garry et al. 1983, Lipschitz 1991) have, however, suggested that factors such as undernutrition, inflammation and pathology may be significant in the etiology of anemia seen in older adults. In the present study, rats were fed nutritionally complete diet,
and dietary VA effect on hematologic and transport iron variables was not significant. Although, inflammation was not systematically evaluated in the current study, there were no signs of gross pathology in the animals. Lesions on hind feet were noted; however, in all old rats (see Dawson et al. 1999). In infections, a decrease in iron in the functional pool is generally associated with a mobilization of iron into stores (Hillman and Finch 1985); in contrast, in the current study, old rats had lower iron stores in primary sites of iron storage, namely the liver, spleen and bone marrow (as discussed below). Moreover, hemoglobin and hematocrit were positively correlated with total liver iron and marrow iron ($r = 0.38–0.50$, $P < 0.04$). Thus, the observations of lower hematologic and transport iron variables in old rats in the present study are most likely related to aging rather than inflammation.

Liver, spleen and bone marrow are the chief sites of iron storage. The effect of aging, from middle-age to old-age, on iron stores in these sites is not fully understood. In the current study, the total iron content of these key tissues involved in iron storage in the body was dramatically lower in old rats (by 59% in liver, 35% in spleen and 38% in femur marrow) than in middle-aged rats. Iron concentration (per gram wet tissue or per gram protein) in liver and spleen was also significantly lower in old Lewis rats. This finding is in contrast to reports of a significant age-related increase in iron concentration in liver of animals; 6-mo compared to 24-mo-old male Fischer 344 rats provided free access to food (Cook and Yu 1998), C57BL/6 male mice from 355 to 900 d (Massie et al. 1983) and 14-mo compared to 30-mo-old male C57BL/6N mice provided free access to food (Sohal et al. 1999). The observation of lower spleen iron concentration in old Lewis rats in the current study is in contrast to the findings of Takeda et al. (1996), who reported a significant effect of age in 3-wk compared to 6-mo-old female Wistar rats fed 15% protein diet. Discrepancies related to the definitions used for aging and old age, species and strain differences across studies and the fact that most studies did not control for potentially confounding variables, such as body weight, in their analyses may contribute to conflicting study findings. For the most part, a significant main effect of age over a wide age range, including young to old animals, may have been interpreted as an age-related increase in iron stores in previous studies. A closer inspection of data presented in adult animals in the reports by Morita et al. (1994) and Sherman et al. (1985), however, shows that iron stores in liver and spleen did not increase significantly in 10-mo compared to 6-mo-old female B10BR mice (Fig. 1 in Morita et al. 1994) and in “aged” (26 mo) compared to “mature” (14 mo) Fischer rats fed control diet containing 16% casein (Figs. 1, 2 in Sherman et al. 1985). Thus, our findings of dramatically lower total iron in liver and spleen as well as lower iron concentration in these tissues are in contrast to most of the previous reports and may be related to the fact that we controlled for the confounding effects of body weight and VA intake as well as in the case of spleen iron variables, in our analyses prior to evaluating age effects.

There is a paucity of information on the effect of aging on hemosiderin iron in bone marrow. After adjusting for body weight and dietary VA, old rats had significantly lower bone marrow smear iron grade as compared to middle-aged rats. Furthermore, a greater proportion of older control rats had reduced to absent hemosiderin iron in bone marrow smears compared to middle-aged control rats (Fig. 1). Because the number of animals in various grades of marrow iron smear was small, this trend could not be evaluated statistically. In contrast, in an earlier report (Benzie 1963) involving necropsies, an age-associated increase in the intensity of iron in sternum marrow smears was noted irrespective of the cause of death. Differences in the models used and in the methods for bone marrow smear preparation may partly account for the differences in our study findings than in those of Benzie (1963).

Bone marrow iron concentration has not been examined in most of the previous studies on iron stores with aging. We examined the age-associated changes in chemical estimates of iron in femur marrow after controlling for significant confounders, body weight and VA intake. Total femur marrow iron and femur marrow iron concentration per gram tissue were significantly lower, by 38 and 59% respectively, in old than in middle-aged rats in the current study. In one report (Takeda et al. 1996), no differences were observed in nonheme iron concentration in marrow obtained from tibia in young (3 wk) and mature (6 mo) female Wistar rats fed control diet (Table 9, 15% protein diet in Takeda et al. 1996). Interestingly, in the present study, when bone marrow iron concentration was expressed per gram protein, no differences were noted between middle-aged and old rats. This suggests that the protein content of bone marrow, reflecting active hematopoietic tissue, was reduced in old rats. Other authors have noted a decrease in the amount of hematopoietic tissue in bone marrow of humans with advancing years (Hartsock et al. 1965). Timaffy (1962) speculated that the active tissue in bone marrow may be replaced by fat deposits with aging, which may be associated with reduced bone marrow responsiveness in older adults.

These observations suggest that total bone marrow iron and active bone marrow mass may be reduced with advancing age, rendering older animals vulnerable to impaired red blood synthesis and subsequent decline in circulating red cell mass and associated functional manifestations of iron deficiency and anemia (Hillman and Finch 1985).

To our knowledge this is the first simultaneous examination of marrow iron stores by chemical estimation of nonheme iron and semiquantitative iron grading of marrow smears. Marrow

<table>
<thead>
<tr>
<th>Liver iron</th>
<th>Spleen iron</th>
<th>Marrow iron</th>
</tr>
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<tbody>
<tr>
<td>$\mu$mol/g wet tissue</td>
<td>$\mu$mol/g wet tissue</td>
<td>$\mu$mol/g wet tissue</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>Hematocrit</td>
<td>Plasma iron, $\mu$mol/L</td>
</tr>
<tr>
<td>$r$</td>
<td>$P$</td>
<td>$r$</td>
</tr>
<tr>
<td>0.43</td>
<td>0.0264</td>
<td>0.54</td>
</tr>
</tbody>
</table>

$^1 n = 29.$
associated functional outcomes of declining iron status with animal models and in humans with advancing age and describe comprehensive evaluation of iron status in Lewis rats, contrary to expectation. In this study, we observed that the bone marrow smear iron evaluation reflects iron status as assessed by biochemical means.

In summary, the findings of reduced iron content in marrow iron smears, lower marrow iron, lower iron stores and iron concentration in liver and spleen, along with lower iron in transport (plasma iron) and functional (hemoglobin and hematocrit) pools, suggest that overall iron status declined in old rats compared to middle-aged rats. Given that animals were fed diets adequate in dietary iron from weaning throughout life, the possible mechanisms for lower hematological status in old Lewis rats in the current study may include reduced iron absorption, increased iron loss, reduced marrow capacity and responsiveness, reduced red blood cell synthesis and/or shortened erythrocyte life span. There are some indications that erythropoiesis may be reduced with aging and that the aging hematopoietic system may have a reduced reserve capacity, making it susceptible to environmental insults that are known to affect the bone marrow adversely (Hartsoc et al. 1965, Lipschitz 1991, Timaffy 1962). These possible mechanisms for observed declines in hematologic variables and iron stores in Lewis rats in the current study need further examination in future studies where inflammation is also systematically examined. Furthermore, there is a need to examine whether there are functional ramifications associated with the age-related decline in hematologic and storage iron variables observed in the present study and whether increasing dietary iron would prevent these changes. Alternatively, whether the biochemical declines in iron parameters represent age-related adaptation(s) also need further investigation. In conclusion, this comprehensive evaluation of iron status in Lewis rats, contrary to usual belief that iron stores increase with aging, body iron was significantly lower in all compartments in old than in middle-aged Lewis rats. Future studies need to examine whether similar findings are observed in other rat strains, animal models and in humans with advancing age and describe associated functional outcomes of declining iron status with advancing age.

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LITERATURE CITED


