Fortification of food with folate began in the United States on January 1, 1998 (1) because of folate’s role in the prevention of neural tube defects (2,3) and the potential association with reduced risk for vascular disease (4) and cancer (5). Canada, Chile, and Israel have more recently started universal mandatory folic acid (FA) fortification (5,6). Public health policies promoting daily intake of FA supplements by women of childbearing age have not resulted in increased supplement use. In contrast, food fortification has been associated with a moderate reduction in neural tube defect rates in the United States and Canada (6). Despite this reduction, universal FA fortification remains controversial because of the concern that additional FA in the diets of certain population groups (e.g., children and the elderly) not initially targeted for fortification may suffer adverse effects, such as the potential masking of vitamin B-12 deficiency, a condition that affects 10–15% of the population >60 y old (6).

FA is considered to be a nontoxic vitamin, but recent data from our group showed a negative effect of high-dose folate supplementation on dietary metabolic protein utilization in pregnant and virgin rats (7–9).

The liver is a key organ in homeostasis. Because of its location between the intestinal mucous and general blood circulation, it receives nutrients, toxins, and agents of infection. Some of these substances induce hepatocyte death, activating regenerative processes that maintain hepatosomatic equilibrium. There is some controversy concerning the effect of aging on hepatocyte fine structure and function, in both humans and rodents (10).

Classical studies showed that hepatic regeneration as well as the total number of hepatocytes decreases with aging, with the disappearance of nearly all mitotic figures in 30-mo-old rats (11). More recently, it was shown that proliferating cell nuclear antigen (PCNA) levels inside the cytoplasm and/or nucleus in hepatocytes of 24-mo-old rats was ~50% lower than that in weanling rats (12). Perhaps, as Popper suggested in 1985 (13), the primary age-related deficit in the liver is reduced adaptive responsiveness, i.e., a diminished ability to respond to a stimulus.

To our knowledge, potential benefits of dietary FA supplementation for hepatic regeneration have not been described. Hepatotrophic activity of FA per se as well as after a partial hepatectomy and hepatic ischemia in rats was reported (14,15), although the mechanisms remain unclear. Other pos-
itive effects of folate or methyl treatments in injured liver include a modulation of hepatotoxicity induced in animal models by carbon tetrachloride (16,17), methotrexate (18), and cyclophosphamide or 5-fluourouracil (19), or protection against the early onset of abnormal hepatic methionine cycle–mediated alcoholic liver disease (20).

There is also a lack of information related to the changes that occur in the liver with age. In fact, we do not know whether the FA needs of distinct tissues and requirements of animals at diverse ages vary.

The present study was conducted to examine the effects of FA supplementation on liver histology, morphology, immunohistochemistry, and biochemical markers in weanling and aged rats compared with controls.

MATERIALS AND METHODS

**Animals and diets.** Male Wistar rats from the Animal Service, Universidad San Pablo-CEU, were divided in two groups: aged [18 mo, considered as aged rats as previously reported (9,21,22)] and weanling (3 wk old) rats. The rats had free access for 4 wk to either a diet supplemented with 40 mg folic acid/kg diet folate (n = 6 rats in the aged supplemented group, and n = 15 rats in the weanling supplemented group) or a control diet that met the rat FA requirements (23), with 1 mg FA/kg diet, (n = 5 rats in the aged control group, and n = 15 rats in the weanling control group), as reported previously (9). Both diets were based on the pure amino acid diet (17% amino acid, Dyets) (24), the most reliable diet treatment for studying the exclusive effect of dietary folic acid, without confounding factors (7–9,17,25–27). On d 29, rats were anesthetized with CO2 and killed by decapitation. The experiment was approved by the Ethical Committee of Universidad San Pablo-CEU.

**Histology.** Livers were rapidly fixed in 10% formalin and kept in 70% alcohol until embedded in paraffin. Thick cuts (5 μm) were stained with histological techniques such as hematoxylin-eosin (H-E), trichromes, sirius red, and reticuline (specific for collagen); 7–10 slides, with 3–4 sections each, were studied.

**Immunohistochemistry.** Immunohistochemistry was performed using monoclonal antibody anti-PCNA (Dako), and cytokeratin-8 (CK-8, Progen Inmuno-Diagnostika, Spain). Three slides with a minimum of 3–4 sections each were studied. The sections were incubated for 10 min with 2% hydrogen peroxide, and then incubated with primary antibody (PCNA 1:100 and CK-8 1:10 dilutions) for 10 min, using Envision (Dako) 1:2 in PBS as the secondary antibody for 30 min. The stain was developed using the 3,3′-diaminobenzidine tetrahydrochloride kit (Dako), following the manufacturer’s instructions.

**Counting of hepatic nuclei.** We used a Nikon Optiphot microscope coupled to a video camera, and connected to a computer provided with image analysis software (Vids IV, Analytical Measuring Systems, version 1.0 for aged rats; MetaMorph, Meta Imaging Series Environmental, version 5.0 rl, Universal Imaging Corporation for weanling rats). H-E stained liver sections from all aged rats and 5 rats from each weanling group were examined. The nuclei of hepatocytes from 100 microscopic fields, 20X magnification, were counted on the computer screen by two different observers (Table 1). The first row was chosen randomly, followed by 99 consecutive rows, without duplication of the counted area. If counting was impeded in 15% of the screen (e.g., end of the section, large vessel, tissue folded, or a hole produced during histological processing), the section was discarded and the next section was examined.

**Labeling index (LI).** To calculate the labeling index (LI), which reflects hepatic mitosis and consequently, hepatic regeneration, anti-PCNA–labeled sections were used. The nuclei were scored as positive or negative with no consideration of staining intensity. In some cases, staining was repeated to achieve homogeneous staining. We followed criteria similar to those for counting of the hepatic nuclei. The LI was calculated as the percentage of the total number of positive PCNA nuclei in the total number of hepatic nuclei in 100 rows.

**Serum biochemical markers.** Whole blood was collected and serum was separated. Folate levels were measured as described previously (27). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, glucose oxidase, creatinine, total bilirubin, and uric acid were measured with a Chemistry Profile Analyzer (CPA) (Coulter CPA, Kemta Cientifica S.A.).

**Statistics.** Results were expressed as means ± SEM. Differences in means were tested by two-way ANOVA. When the ANOVA was significant, multiple comparisons between means were made using Tukey’s test. Differences were considered significant at P < 0.05 (SYSTAT Version 5.0).

RESULTS

Routine histological staining of all livers showed portal triads formed by a great vein with thin walls, accompanied by two hepatic conducts or bile ducts, easily identified because of the enlarged and dark cells of the wall. Hepatic arteries were small and the nuclei of the endothelial cells were prominent in the inner vascular light, with abundant surrounding smooth muscle. No anatomical pathologial signals were found in any of the specimens.

The total number of hepatocytes in aged control rats tended to be 17% lower (P = 0.07) than in aged, supplemented rats (Table 1), which did not differ from either group of weanling rats. The difference in the number of hepatocytes.

### TABLE 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet FA n</th>
<th>Hepatic nuclei</th>
<th>PCNA positive nuclei</th>
<th>LI³</th>
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<tr>
<td></td>
<td>mg/kg</td>
<td>n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weanling</td>
<td>40</td>
<td>5</td>
<td>18,224 ± 492.9</td>
<td>7913.6 ± 988.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>17,871 ± 338.7</td>
<td>8600.3 ± 951.2</td>
</tr>
<tr>
<td>Aged</td>
<td>40</td>
<td>6</td>
<td>18,005 ± 352.7</td>
<td>330.0 ± 159.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>14,995 ± 533.9</td>
<td>198.4 ± 64.8b</td>
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</table>

ANOVA

<table>
<thead>
<tr>
<th>Age</th>
<th>FA</th>
<th>Age × FA</th>
<th>P &lt; 0.001</th>
<th>P &lt; 0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Means in a column without a common letter differ, P < 0.05.
2 Hepatic nuclei: 100 microscopic fields, 20X magnification, from H-E stained sections were counted on the computer screen.
3 Labeling index = (number of PCNA positive nuclei from 100 anti-PCNA labeled files/number of hepatic nuclei) × 100.
was also demonstrated in tissue sections of aged rats that were evaluated at high-power magnification; hepatocytes were larger and had bigger nuclei in controls than in FA-supplemented diets (Fig. 1A and B).

Supplementation with FA did not alter the number of PCNA positive nuclei but the number was lower in aged than in weanling rats (Table 1). PCNA staining showed that in both groups of weanling rats, dividing hepatocytes were around the central veins, with no evident differences in their number or disposition. Hepatocytes were rarely dividing in aged rats of either diet group. Positive nuclei were widely dispersed in the tissue with no specific areas of location (Fig. 1C and D).

As CK-8 staining showed, bile ducts were abundant in livers from the aged, control rats. The bile duct cells formed cholestatic rosettes, with a cauliflower-like appearance inside the hepatic parenchyma (Fig. 1F). On the contrary, in livers of the aged, supplemented rats, the bile ducts were straight and in higher proportion than the cholestatic rosettes (Fig. 1F) and resembled livers of the weanling rats in which bile ducts were less abundant and straight in both diet groups. PCNA staining also showed that in aged, control rats, the bile duct cells were actively proliferating (Fig. 1F and G).

As expected, supplementation with FA increased serum folate concentrations (P < 0.001) in both weanling (591.8 ± 5.9 vs. 192.2 ± 31.6 nmol/L in controls) and aged rats (445.7 ± 7.3 vs. 65.7 ± nmol/L in controls). FA supplementation did not alter serum AST, ALT, urea, glucose oxidase, total bilirubin, or uric acid. However, serum creatinine concentrations were lower (P < 0.05) in weanling supplemented (12.44 ± 1.25 μmol/L) compared with weanling control rats (16.71 ± 1.36 μmol/L). Age, regardless of dietary FA level, lowered serum AST (172 ± 20.3 in aged rats vs. 302 ± 16.3 U/L in weanling rats, P < 0.005) and ALT (98.8 ± 11.9 in aged rats vs. 128 ± 5.9 U/L in weanling rats, P < 0.005). Serum creatinine concentrations were greater (P < 0.001) in aged rats (33.68 ± 5.84 μmol/L) than in weanling rats (14.58 ± 2.61 μmol/L).

DISCUSSION

Five years have passed since the fortification program began in the United States. This indiscriminate fortification includes population groups that do not require fortification either because they do not have cardiovascular problems or because they will not become pregnant (e.g., men, postmenopausal women). Members of these groups may eventually consume more than the optimal amount of FA. Typical intakes of FA from fortified foods are more than twice the level originally intended, which was an average increase in FA intake of 100 μg/d (28,29).

PCNA is widely used as a quantitative measure of hepatic regenerative activity, in humans and rats (12,30–32). Hepatic regeneration in normal rats declines with age (11,12). In the present study, FA supplementation tended to stimulate regenerative capacity in livers of the 18-mo-old rats (17% more hepatocytes) with no effect in weanling rats. It was reported that liver PCNA staining in 24-mo-old Wistar rats differentiated benign from malignant parenchyma tumors (31), as indicated by LI values in normal livers, hepatocellular adenomas, hepatocellular carcinomas, and cystic cholangiomas. However, given that values in normal livers and hepatocellular adenomas overlapped, with LI between 0.74 and 0.96, a histological study of sections is critical. On the basis of their criteria, our LI values are high; thus, the rats would be considered to have hepatocellular adenoma. However, the histological study, which was based mainly on a number of sections comparable to biopsy specimens, showed aleatory distribution of positive nuclei, which excluded adenomatous formations that are characterized by proximity. We postulate that FA supplementation enhances hepatocyte proliferation.

Folate status affects cell proliferation (33). Abnormal cell proliferation is involved in carcinogenesis, including the pro-

![FIGURE 1](https://academic.oup.com/jn/article-abstract/134/5/1130/4688542) Histology and immunohistochemistry in weanling and aged male Wistar rats fed FA-supplemented (40 mg/kg diet) or control (1 mg/kg diet) diets for 4 wk. (A, B) H-E staining. The density of hepatic nuclei density was lower in livers of aged, control rats (panel A) than in livers of aged, supplemented rats (panel B). (C, D) Anti-PCNA staining in aged rats. Positive hepatic nuclei were scarce in livers of aged control rats (panel C). In liver of aged, supplemented rats (panel D), they were more numerous and widely dispersed in the tissue, with no specific areas of location. (E, F) Anti-CK-8 staining. Bile ducts were abundant and formed cholestatic rosettes in livers of aged, control rats (panel E). In liver of aged, supplemented rats (panel F), they were straight and in higher proportion. (G, H) Anti-PCNA staining in aged rats. Bile ductal cells were actively proliferating in livers of aged, control rats (panel G). In liver of aged, supplemented rats (panel H), dividing duct cells were scarce. B: bile duct; P: portal vein. Arrows: Positive bile cells. Scale bar: 10 μm for A and B, 25 μm for C and D, 50 μm for E, F, G, and H.
cesses of initiation and promotion, although its precise role has not been clarified (34). Folate status, in many situations, modifies proliferation rates, increasing proliferation in the livers of folate- or methyl-deficient rats (35). On the other hand, folate supplementation can reduce carcinogen-induced ornithine decarboxylase and tyrosine kinase activities, both indices of cellular proliferation (36). Recently, a human colon cancer cell line in which folate derivatives had growth-inhibitory activity was reported (37).

FA administration induced hepatic regeneration per se and also following ischemia and ischemia plus partial hepatectomy in adult male rats, by increasing the percentage of regenerating hepatocytes (14, 15). The mechanism through which FA exert its hepatoprotective activity is unknown, but some hypotheses have been suggested. After partial hepatectomy, the stores of FA diminish, whereas DNA synthesis, and therefore, FA requirements, increase rapidly. In this context, the availability of the folate compounds might limit DNA synthesis in both velocity and intensity. For the hepatoprotective activity of FA per se, it was proposed that the transmethylating ability of FA could result in changes in the biochemical structure of the target molecules involved in DNA synthesis.

In our study, dietary FA supplementation of 18-mo-old rats tended to increase hepatocyte division and improve liver morphology. Future studies will be necessary to corroborate these results and to identify the underlying mechanisms.

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LITERATURE CITED