ORIGINAL PAPER

Reiko Meguro · Yoshiya Asano · Hiroyasu Iwatsuki ·
Kazuhiko Shoumura

Perfusion-Perls and -Turnbull methods supplemented by DAB intensification for nonheme iron histochemistry: demonstration of the superior sensitivity of the methods in the liver, spleen, and stomach of the rat

Abstract Perfusion-Perls and -Turnbull methods supplemented by the intensification with 3,3′-diaminobenzidine (+ DAB) enabled stronger and more extensive staining of nonheme iron than the Perls + and Turnbull + DAB methods carried out on tissue sections fixed with 10% formalin in 0.9% saline or PBS. The section- and perfusion-Perls + DAB methods are not specific for the demonstration of nonheme ferric iron but also stain nonheme ferrous iron. However, owing to its high sensitivity, the perfusion-Perls + DAB method would provide useful information about nonheme iron deposition regardless of oxidation states in normal and pathological conditions. The perfusion-Turnbull + DAB method is specifically demonstrable of nonheme ferrous iron and the results from this method showed significant stores of nonheme ferrous iron in the hepatocytes, Kupffer cells, splenic macrophages, and gastric parietal cells of the rat. Since nonheme ferrous iron is considered to be critically involved in free radical generation, the perfusion-Turnbull + DAB method would visualize such populations of cells that are at risk from free radical damage.

Keywords Nonheme iron · Histochemistry · Perfusion-Perls method · Perfusion-Turnbull method · DAB intensification

Introduction

Iron is one of the essential trace metals of the body, where it is present in the form of heme or nonheme. Heme is iron protoporphyrin complex that works as a reactive center for many heme enzymes and the respiratory chain of mitochondria. Nonheme iron is present in a variety of iron–protein complexes including the storage and sequestration proteins (ferritin and hemosiderin; Crichton and Ward 1992), proteins of iron transport (ferritransferrin; Crichton and Ward 1992), nonheme iron enzymes (for example, ribonucleotide reductase; Fontecave 1998), nonheme iron–sulfur enzymes and proteins (for example, aconitase, NADP dehydrogenase, and ferredoxins; Beinert et al. 1996; Cotton et al. 1999), and low molecular weight iron species in which iron is loosely bound to low molecular weight organic substances (for example, citrate, AMP, ATP, various amino acids and peptides; Crichton and Ward 1992).

Because of ubiquitous distribution and high reactivity, heme and nonheme iron interaction with free radicals and nitric oxide has been considered to play a central role in various physiological and pathophysiological conditions. In ischemia and reperfusion of organs, Fe^{2+} liberated from low molecular weight nonheme iron species and Fe^{2+} (and Fe^{3+}) released from ferritin are considered to catalyze the production of cell-injuring hydroxyl radicals via the Fenton reaction (Meneghini 1997). Furthermore, altered iron homeostasis has been emphasized as a cause of neurodegenerative diseases such as Parkinson's, Alzheimer's, Huntington's, and other diseases (Gerlach et al. 1994). The nitric oxide interaction with heme and nonheme iron is involved in signal transduction and inhibition of mitochondrial respiration and DNA synthesis (Gross 1995; Cooper 1999).

For the experimental and pathological investigations of the disorders in which free radical interaction with iron and altered iron homeostasis have been implicated, iron histochemistry using the Perls technique has been the method of first choice. This method is excellent in imaging capability, universally available, and inexpensive, although the minimum detection limit for nonheme iron is very high (Perl and Good 1992). To improve the

R. Meguro (✉) · Y. Asano · H. Iwatsuki · K. Shoumura
Department of Anatomy,
Hirosaki University School of Medicine,
036-8562 Hirosaki, Japan
e-mail: meguro@cc.hirosaki-u.ac.jp
Tel.: +81-172-395002
Fax: +81-172-334540

sensitivity of the Perls method, intensification with 3,3′-diaminobenzidine (DAB; Nyguen-Legros et al. 1980) and post-DAB enhancement (Moos and Møllgård 1993) were introduced.

We present here two simple and sensitive methods for the visualization of nonheme ferric and ferrous iron [Fe (III) and Fe (II)]: the perfusion-Perls and -Turnbull methods supplemented by DAB intensification (+ DAB). The liver, spleen, and stomach of the rat were used to compare the results of the perfusion-Perls + DAB and perfusion-Turnbull + DAB methods with those of the section-Perls + DAB (the method of Nyguen-Legros et al. 1980) and section-Turnbull + DAB methods performed on formalin-fixed paraffin sections. Some results of application of the perfusion-Turnbull + DAB method to the ischemic cat brains appeared in a previous paper from this laboratory (Yu et al. 2001).

Materials and methods

Animal treatment and perfusion fixation of tissues

Ten female Wistar rats (body weight 202–264 g) were used. The animals were supplied from the Institute of Animal Experiments, Hirosaki University School of Medicine. The animals were maintained with free access to food (MF; Oriental Yeast, Chiba, Japan) and water, which contained 154 μg/g iron and 0.07 μg/ml iron, respectively; the animals were neither iron-deficient nor iron-overloaded. All animal experiments in this study followed the Guidelines for Animal Experimentation, Hirosaki University.

The animals were deeply anesthetized with intraperitoneal pentobarbital sodium (38–50 mg/kg), a thoracotomy and a left ventricular incision were made to perfuse the fixatives described below through the ascending aorta, and the right auricle was incised to flush out blood and fixatives. All perfusions were initiated within 4 min after thoracotomy and within 1 min after cardiotomy and performed at the rate of 20 ml/min. The liver, spleen, stomach, and other organs were removed 1 h after perfusion except for rats 4 and 8 (Table 1) in which the organs were removed after overnight fixation in situ. The excised tissues were then dehydrated in graded ethanol, embedded in paraffin, cut at 5 μm thickness, and mounted on silane-coated glass slides (Matsunami, Osaka, Japan). In addition, all aqueous solutions described below were prepared with ion-exchanged distilled water.

For the section-Perls + DAB and section-Turnbull + DAB methods performed on paraffin sections, two rats were perfused with 10% formalin (Wako, Osaka, Japan) in 0.9% saline (formalin–saline, pH 3.7, rat 1; Table 1) or with 10% formalin in phosphate-buffered saline (formalin–PBS, pH 7.2, rat 2; Table 1).

For the perfusion-Perls + DAB method, four rats (rats 3–6; Table 1) were perfused with the solution (pH 0.5–0.6, 1,000 ml) containing 1% HCl (Wako), 1% potassium ferrocyanide [potassium hexacyanoferrate (II), lemon yellow crystals or powder; Wako], and 10% formalin. A PBS flush (pH 7.4, 150 ml) and formalin–PBS (150 ml) perfusion were carried out before the perfusion-Perls method in rats 5 and 6, respectively. For the perfusion-Turnbull + DAB method, four rats (rats 7–10; Table 1) were perfused with the solution (pH 0.5–0.6, 1,000 ml) containing 1% HCl, 1% potassium ferricyanide [potassium hexacyanoferrate (III), ruby-red crystals or powder; Wako], and 10% formalin. Initial PBS flush (150 ml) and formalin–PBS perfusion (150 ml) were made in rats 9 and 10 (Table 1), respectively.

Section treatment

Section-Perls + DAB and section-Turnbull + DAB methods

Sections taken from the liver, spleen, and stomach of rats 1 and 2 (Table 1) were divided into two groups; each group contained sections from each organ described above. Each group of sections was dewaxed–hydrated, incubated in the solution (pH 0.5–0.6, 25°C) containing 1% HCl and 1% potassium ferrocyanide or in the solution (pH 0.5–0.6, 25°C) containing 1% HCl and 1% potassium ferricyanide for 30 min, and washed in distilled water.

Sections of both groups were then incubated for 30 min in 25°C methanol (Wako) containing 0.3% H_2O_2 (Wako) and 0.01 M NaN_3 (Wako) to inhibit peroxidase and catalase activities and to eliminate the pseudoperoxidase activity in the erythrocytes (Straus 1971; Pool et al. 1983; Feng et al. 1998). After washing in 0.1 M phosphate buffer (PB, pH 7.4) sections were treated with 0.025% 3,3′-diaminobenzidine–4HCl (DAB; Sigma, St. Louis, Mo., USA) and 0.005% H_2O_2 in 0.1 M PB (pH 7.4, 25°C) for 30 min (DAB intensification; Nyguen-Legros et al. 1980) and counterstained with hematoxylin. The DAB intensification was omitted in several additional sections from each organ.

In addition, sections of the spleen of rat 2 were used to study staining variability depending on pH of the solution for iron histochemistry. Mixtures of different pH were prepared by changing the ratio of the volumes of 2% potassium ferrocyanide and 2% HCl: 1:1, 10:1, 20:1, 40:1, 80:1, 100:1, and 1:0 mixtures were pH 0.6, 2.2, 3.0, 3.6, 3.8, 4.3, and 6.9, respectively. Sections were then processed for DAB intensification after treatment with 0.3% H_2O_2–methanol and 0.01 M NaN_3.

DAB intensification after iron histochemistry by perfusion-Perls and -Turnbull methods

Sections were dewaxed–hydrated, pretreated with 0.3% H_2O_2–methanol (25°C) containing 0.01 M NaN_3 for 30 min, and intensified with 0.025% DAB and 0.005% H_2O_2 in 0.1 M PB (pH 7.4, 25°C) for 30 min. After washing in distilled water, sections were counterstained with hematoxylin. Several additional sections were stained with hematoxylin without DAB intensification.

In addition, some chemical experiments were undertaken to test the specificity of Perls and Turnbull reactions, using $FeCl_2$, $FeCl_3$, $ZnCl_2$, and $CuCl_2$ purchased from Wako.

Results

The perfusion-Perls and -Turnbull methods stained non-heme iron accumulating within organs and tissues deep to light blue (perfusion-Perls method) or bluish green (perfusion-Turnbull method), which enabled easy identification of the organs and tissues containing iron. The DAB intensification of Perls and Turnbull reactions made such populations of cells visible that were only palely stained or unstained in unintensified sections.

Staining by section-Perls + DAB method

There were some differences in the results (Table 1) between the tissues (rat 1) perfused with formalin-saline and those (rat 2) perfused with formalin–PBS. The liver fixed with formalin–PBS showed more intensely stained hepatocytes (Fig. 1C) than that fixed with formalin–saline (Fig. 1A). In both formalin–saline and formalin–PBS fixations, macrophages were intensely stained in the red

75

Table 1 Summary of fixation, histochemistry, and observations in ten rats. (*DAB* DAB intensification, *overnight* overnight fixation, + lightly stained, ++ moderately stained, +++ intensely stained)

| Rat number	Fixative	Histochemistry	Liver	Spleen Red pulp	White pulp and marginal zone	Stomach
1	10%Formalin–saline	Section-Pedrls& + DAB Section-Turnbull& + DAB	Hepatocytes (+), Kupffer cell (−); Fig. 1A Unstained; Fig. 1B	Macrophages (+++); Fig. 2A Macrophages (−); Fig. 2B	Several macrophages (±), fragmentary; Fig. 2A Unstained; Fig. 2B	Unstained; Fig. 3A Unstained; Fig. 3B
2	10%Formalin–PBS	Section-Pedrls& + DAB Section-Turnbull& + DAB	Hepatocytes (++), Kupffer cell (−); Fig. 1C Unstained; Fig. 1D	Macrophages (+++); Fig. 2C Macrophages (+); Fig. 2D	Macrophages (++ ~ +++), fragmentary; Fig. 2C Unstained; Fig. 2D	Unstained; Fig. 3C Unstained; Fig. 3D
3	Perfusion-Pedrls	DAB	Hepatocytes (++), Kupffer cell (−)	Macrophages (+++); Fig. 2E	Macrophages (+++); Fig. 2E	Parietal cells (+ ~ ++)
4	Perfusion-Pedrls/ overnight	DAB	Same as rat 3	Same as rat 3	Same as rat 3	Same as rat 3
5	Perfusion-Pedrls after PBS	DAB	Hepatocyte (++), Kupffer cell (+++); Fig. 1E	Same as rat 3	Same as rat 3	Same as rat 3
6	Perfusion-Pedrls after formalin–PBS	DAB	Hepatocyte (++), Kupffer cell (+++)	Same as rat 3	Same as rat 3	Same as rat 3; Fig. 3E
7	Perfusion-Turnbull	DAB	Hepatocyte (++), Kupffer cell (+++)	Macrophages (+++); Fig. 2F	Macrophages (++); Fig. 2F	Parietal cells (+ ~ ++)
8	Perfusion-Turnbull/ overnight	DAB	Hepatocyte (++), Kupffer cell (−)	Same as rat 7	Same as rat 7	Same as rat 7
9	Perfusion-Turnbull after PBS	DAB	Same as rat 7	Same as rat 7	Same as rat 7	Same as rat 7
10	Perfusion-Turnbull after formalin–PBS	DAB	Same as rat 7	Same as rat 7	Same as rat 7	Same as rat 7; Fig. 3F
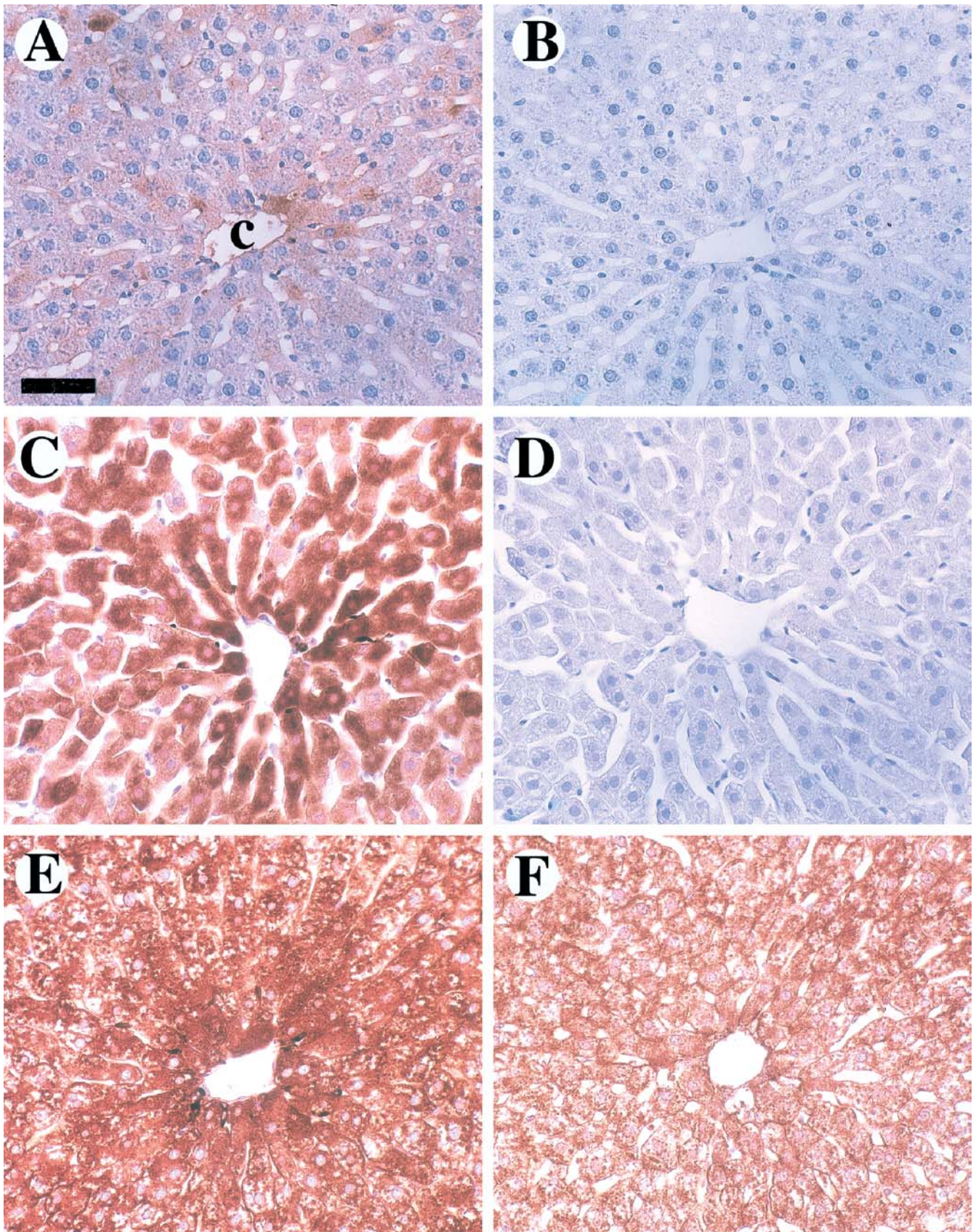
Fig. 1A–F Liver of rat 1 (**A**, **B**), rat 2 (**C**, **D**), rat 5 (**E**), and rat 9 (**F**). **A** Section-Perls method after formalin–saline. **B** Section-Turnbull method after formalin–saline. **C** Section-Perls method after formalin–PBS. **D** Section-Turnbull method after formalin–PBS. **E** Perfusion-Perls method after PBS. **F** Perfusion-Turnbull method after PBS. *c* Central vein. *Bar* 50 μm
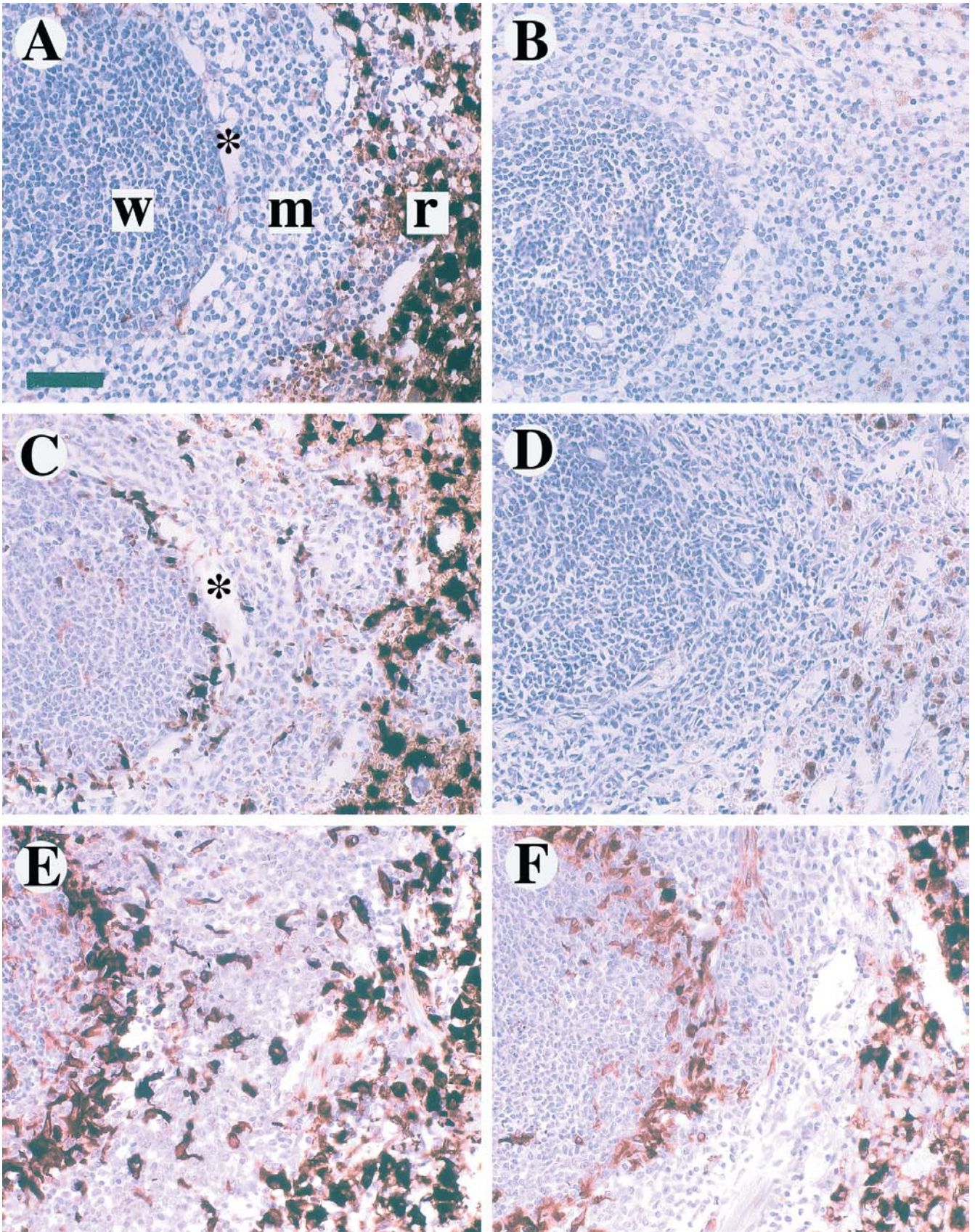
Fig. 2A–F Spleen of rat 1 (**A**, **B**), rat 2 (**C**, **D**), rat 3 (**E**), and rat 7 (**F**). **A** Section-Perls method after formalin–saline. **B** Section-Turnbull method after formalin–saline. **C** Section-Perls method after formalin–PBS. **D** Section-Turnbull method after formalin– PBS. **E** Perfusion-Perls method. **F** Perfusion-Turnbull method. *m* Marginal zone, *r* red pulp, *w* white pulp. *Asterisks* indicate the marginal sinus. *Bar* 50 μm
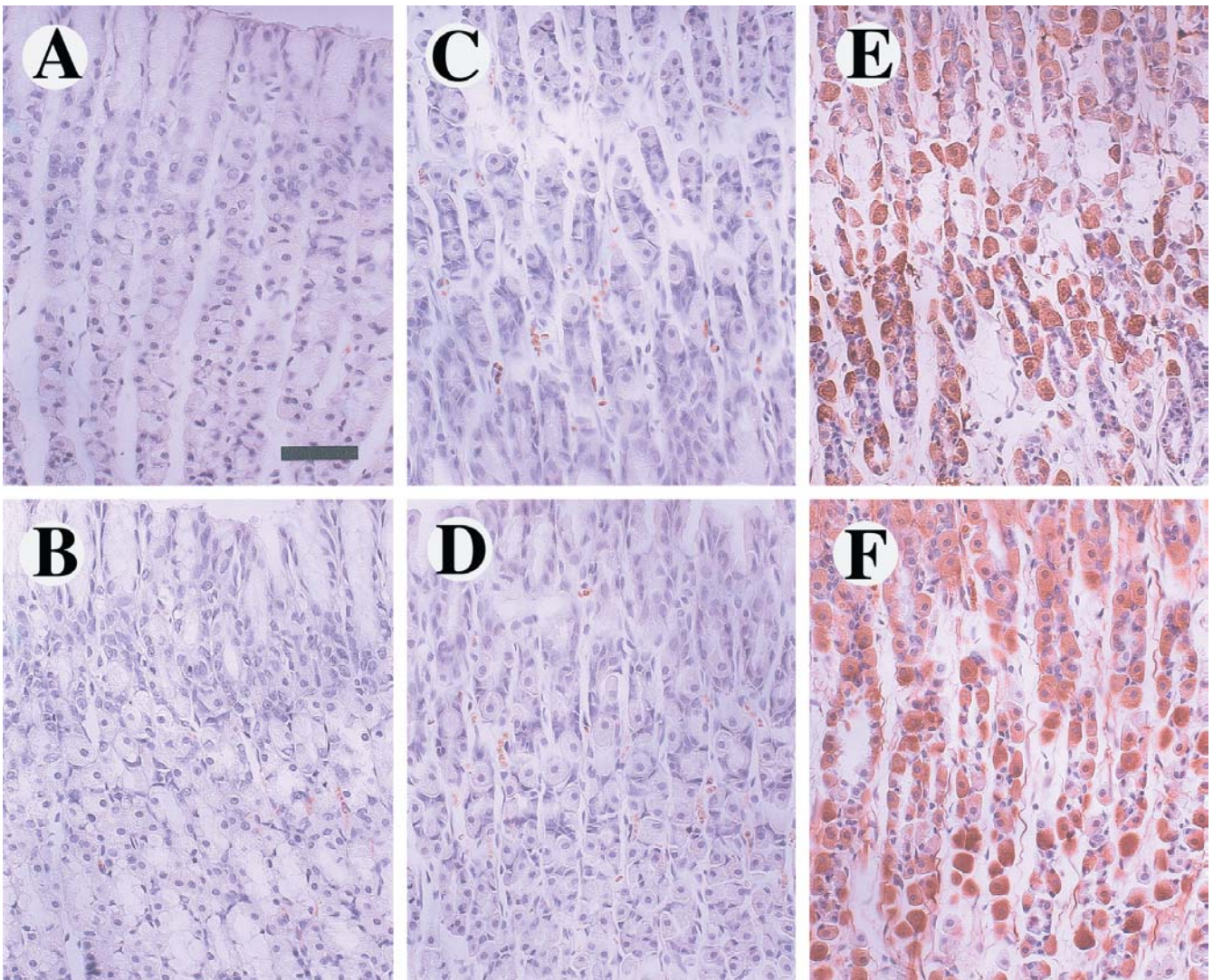
Fig. 3A–F Stomach of rat 1 (**A, B**), rat 2 (**C, D**), rat 6 (**E**), and rat 10 (**F**). **A** Section-Perls method after formalin–saline. **B** Section-Turnbull method after formalin–saline. **C** Section-Perls method after formalin–PBS. **D** Section-Turnbull method after formalin–PBS. **E** Perfusion-Perls method after formalin–PBS. **F** Perfusion-Turnbull method after formalin–PBS. In each photograph, the gastric mucosal surface is toward the *top*. *Bar* 50 µm

pulp of the spleen (Fig. 2A, C), where the reaction product coalesced into large masses in the cytoplasm. They appeared as prominent blue masses in non-intensified sections. The morphology and distribution of the masses suggested they were hemosiderin. Many macrophages at the margin of the white pulp and in the marginal zone were moderately to intensely stained in the spleen fixed with formalin–PBS (Fig. 2C) whereas only a small number of such cells were very palely stained in the spleen fixed with formalin–saline (Fig. 2A). The morphology of macrophages in the white pulp and marginal zone (Fig. 2A, C) was very fragmentary compared with that visualized by the perfusion-Perls + DAB method (Figs. 2E, 4B). The macrophages aggregated to form a ring around the margin of the white pulp appeared to correspond with marginal metalophils (Snook 1964). Formalin–saline- and formalin–PBS-fixed stomachs

showed almost no staining (Fig. 3A, C) except for a small number of lightly stained red blood cells.

As mentioned in Materials and methods, sections of the formalin–PBS-fixed spleen (rat 2) were treated with Perls solutions prepared for pH 0.6, 2.1, 2.7, 3.4, 3.8, 4.3, and 6.9, and then intensified with DAB. Sections were intensely stained in pH 0.6, 2.1, and 2.7 solutions, lightly stained in pH 3.8 and 4.3 solutions, but unstained in pH 6.9 solution. This indicates that some loosely bound nonheme iron is releasable in weakly acidic solutions, which are comparable to the internal environment of endosomes (pH 5.0–5.5) where Fe^{3+} is liberated from transferrin (Crichton and Ward 1992).

Staining by section-Turnbull + DAB method

There was no staining in the tissues which were treated by the section-Turnbull + DAB method after formalin–saline (Figs. 1B, 2B, 3B; rat 1) or formalin–PBS (Figs. 1D, 3D; rat 2) fixation, except that hemosiderin masses of macrophages were lightly stained in the spleen fixed with formalin–PBS (Fig. 2D). The light brown pigment in the macrophages of the red pulp in Fig. 2B is hemosiderin, which was less prominent or indiscernible in the white pulp and marginal zone.

Staining by perfusion-Perls + DAB method

The results of the perfusion-Perls + DAB method were quite similar regardless of different means of fixation, i.e., with or without initial flush of PBS, with or without initial formalin–PBS perfusion, and with or without overnight fixation (Table 1). The perfusion-Perls + DAB method stained hepatocytes with moderate intensity (Fig. 1E) which was comparable to those stained by the section-Perls + DAB method after formalin–PBS fixation (Fig. 1C). A small number of intensely stained Kupffer cells were observed among hepatocytes in the perivenous area of the hepatic lobule in rats 5 and 6 (Fig. 4A; Table 1). Splenic macrophages were intensely stained in the white pulp, marginal zone, and red pulp (Fig. 2E), and the black reaction product clearly demonstrated the characteristic morphology of the cells with large irregularly shaped cell bodies and short and stout dendrites (Fig. 4B). Hemosiderin masses were almost masked by intense staining of the entire cytoplasm. The perfusion-Perls + DAB method lightly to moderately stained parietal cells of the stomach (Fig. 3E), which were easily identified by large round to pyramidal cell bodies with distinct round nuclei. Almost all parietal cells present in sections were stained; parietal cells at the depth of the gastric glands were more intensely stained than those of the neck region. Parietal cell bodies contained numerous small dark brown granules scattered throughout the cytoplasm (Fig. 4C).

Staining by perfusion-Turnbull + DAB method

The results of the perfusion-Turnbull + DAB method were essentially the same regardless of different means of fixation, i.e., with or without initial flush of PBS, with or without initial formalin–PBS perfusion, and with or without overnight fixation (Table 1). The results were comparable to those of perfusion-Perls + DAB method except that hepatocytes, Kupffer cells (a few cells were stained in rats 9 and 10; Table 1), splenic macrophages, and gastric parietal cells were slightly less intensely stained than those by the perfusion-Perls + DAB method.

The results of chemical experiments to test the specificity of Perls and Turnbull reactions are described and considered in the Discussion.
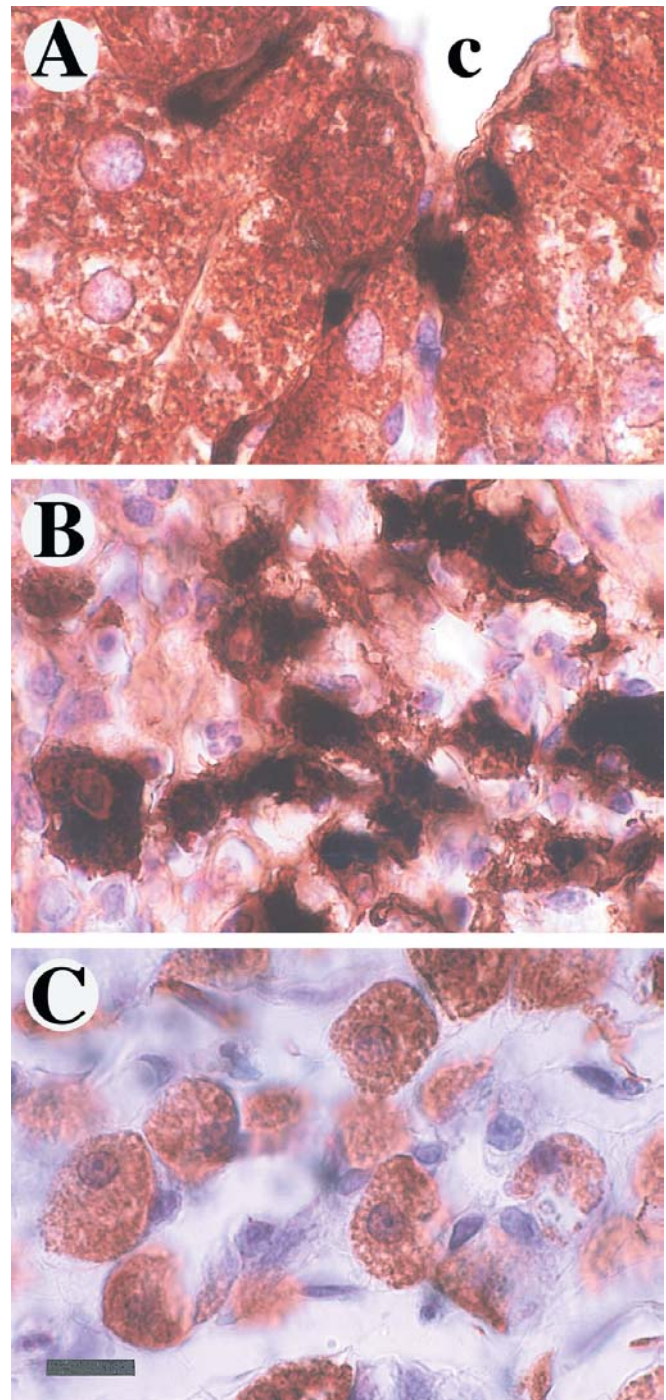
Fig. 4 A Liver of rat 5. A small number of Kupffer cells are intensely stained among moderately stained hepatocytes containing numerous small dark brown granules. *c* Central vein. **B** Spleen of rat 3. Characteristic morphology of macrophages is visible by the black reaction. **C** Stomach of rat 6. Parietal cell cytoplasm contains numerous small dark brown granules. *Bar* 10 μm

Discussion

Among classic methods for nonheme iron histochemistry, the Perls and Turnbull methods are most commonly used (Pearse 1985). The Prussian blue reaction of the Perls

method depends on the production of insoluble precipitate when ferrocyanide ions, $[Fe^{II}(CN)_6]^{4-}$, react with Fe^{3+} liberated in acidic solution. The insoluble blue reaction product of the Turnbull method is formed by reaction of ferricyanide ions, $[Fe^{III}(CN)_6]^{3-}$, with Fe^{2+}. This method has been usually used, not in its simplest form, to stain nonheme Fe (III), after reducing it with $(NH_4)_2S$ (Tirmann-Schmelzer method; Pearse 1985). The simplest form of the Turnbull method has rarely been used because iron in tissues has long been believed to be present almost invariably in ferric form (Pearse 1985). Therefore, there are only a few works where the Turnbull method was used to stain nonheme Fe (II) in tissues. For example, in formalin-fixed sections of the human brain, Morris et al. (1992) observed a positive Turnbull reaction only in the region where the highest reactivity for nonheme iron was obtained by the Perls method.

In the present study, the section-Perls + DAB and section-Turnbull + DAB methods stained nonheme iron only in such cells as splenic macrophages and hepatocytes where higher concentrations of nonheme iron were accumulated. The difference in the results between the section-Perls + DAB method after formalin–saline fixation and that after formalin–PBS fixation can be explained by the difference in pH of the fixatives; the pH of formalin–saline was 3.7 and that of formalin–PBS was 7.2. It is very probable that loosely bound nonheme iron would be released by weakly acidic formalin–saline and washed out during perfusion, leaving a demonstrable amount of iron in the cells where it was densely accumulated. In agreement with the present results, O'Connell et al. (1985) presented evidence suggesting iron release from ferritin and hemosiderin at pH 4.5 even in the absence of a reducing agent. Furthermore, the difficulty of visualizing nonheme Fe (II) by section-Turnbull method + DAB after formalin–PBS fixation strongly suggests inevitable oxidation of nonheme Fe (II) to Fe (III) mainly by oxygen in aqueous solutions and air during tissue preparation and histochemical analysis. We consider this is the most probable reason the ubiquitous distribution of nonheme Fe (II) has long escaped attention. Further, it can be pointed out that the section-Perls method has an advantage of visualizing both nonheme Fe (III) and Fe (II) at the same time for the reason described above.

The section-Perls method after formalin–PBS fixation stained hepatocytes and splenic macrophages but did not stain gastric parietal cells. This suggests that some loss of loosely bound nonheme Fe (III) is inevitable during tissue treatment, unless it turns insoluble iron compounds in advance.

On the other hand, the perfusion-Perls and -Turnbull methods depend on forming insoluble compounds of nonheme Fe (III) and Fe (II) before tissue treatment. The insoluble blue precipitates of the Perls and Turnbull reactions are commonly formulated as $Fe^{III}_4 [Fe^{II}(CN)_6]_3 \cdot 15H_2O$ (Cotton et al. 1999), which is obtained on mixing Fe^{3+} and ferrocyanide ion or on mixing Fe^{2+} and ferricyanide ion. However, there are crossreactions between ferricyanide ion and Fe^{3+} and between ferrocyanide ion and Fe^{2+}. Ferricyanide ion and Fe^{3+} gave a reddish brown solution but no precipitate. Furthermore, the result was the same when $FeCl_3$ was dissolved in the perfusion-Turnbull solution composed of 1% potassium ferricyanide, 1% HCl, and 10% formalin. Thus, the reaction product by the Turnbull method is not contaminated with iron derived from nonheme Fe (III). Ferricyanide ion also reacted with Zn^{2+} and Cu^{2+} to give yellow and green precipitates in acidic solution, respectively, which however quickly dissolved in 0.1 M PB (pH 7.4). Taken together these results indicate that the perfusion-Turnbull + DAB method is highly specific for nonheme Fe (II).

Ferrocyanide ions and Fe^{2+} gave insoluble white precipitate, potassium ferrous ferrocyanide {Everitt's salt, $K_2Fe^{II}[Fe^{II}(CN)_6]$}, the reduced form of Prussian blue (Cotton et al. 1999). Potassium ferrous ferrocyanide was quickly oxidized to Prussian blue by oxygen in solutions and air. As early as 1910, Nishimura used this oxidative production of Prussian blue in his method to stain nonheme Fe (III) after reducing it with $(NH_4)_2S$. Here, it should be emphasized again that the Perls method applied on sections and by perfusion is not specific for nonheme Fe (III) but would stain both Fe (III) and Fe (II) simultaneously. Ferrocyanide ions also reacted with Zn^{2+} and Cu^{2+} giving white and dark brown precipitates, respectively, in a wide range of pH. These precipitates, however, did not catalyze the oxidative polymerization of DAB by H_2O_2. Furthermore, we never observed dark brown precipitate suggestive of the reaction product formed by Cu^{2+} and ferrocyanide ions in the tissues treated by the perfusion-Perls method without DAB intensification.

Nonheme iron stored as ferritin is essentially in the form of Fe (III) and its reduction by various organic substances (for example, ascorbate), nitric oxide, and superoxide is required for iron mobilization from ferritin in physiological and pathophysiological conditions (O'Connell et al. 1985; Thomas et al. 1985; Thomas and Aust 1986; Monteiro et al. 1989). The reductive release of Fe^{2+} from ferritin is unlikely in the present histochemistry because the section-Turnbull + DAB method carried out on formalin–PBS-fixed sections did not stain hepatocytes (Fig. 1D) where ferritin is present in the cytosol and lysosomes (Cooper et al. 1988), whereas the section-Perls + DAB method stained them with moderate intensely (Fig. 1C). In line with this, iron release from ferritin without reducing agents has been noted in weakly acidic solutions (O'Connell et al. 1985).

The other form of iron sequestration, hemosiderin, has trivalent iron cores similar to the ferrihydrite, $Fe^{III}O(OH) \cdot H_2O$, cores of ferritin (Crichton and Ward 1992; Cotton et al. 1999). Interestingly, hemosiderin was stained by the section- and perfusion-Turnbull methods as well as by the section- and perfusion-Perls methods. At present we have no data to explain these results, but it is noteworthy that macrophages generate superoxide and

nitric oxide, which would reduce nonheme Fe (III) to Fe (II) (Cunha et al 1993; Chisolm et al. 1999).

In the cat brain, nonheme iron is stored preferentially in the cytoplasm of oligodendrocytes (Yu et al. 2001). It was stained by section- and perfusion-Perls + DAB methods but unstained by perfusion-Turnbull + DAB method, which meant that cytoplasmic nonheme iron was mostly in the trivalent form (Yu et al. 2001). After 20 min global ischemia of the cat brain, perfusion-Turnbull + DAB method stained the cytoplasm of oligodendrocytes, indicating that nonheme Fe (III) was reduced to Fe (II). On the other hand, the organs were virtually non-ischemic in the present experiments, because fixative perfusion was initiated within 4 min after thorachotomy and 1 min after cardiotomy except rats 5 and 9 which received PBS flush for 7.5 min before fixation (Table 1).

Staining of heme iron is unlikely in the present histochemistry, because treatment with a strong oxidant such as ammonium peroxydisulfate (Okamoto 1937) or 30% H_2O_2 (Pearse 1985) is required to liberate iron from heme; dilute HCl used in this study is not an oxidant but an acid which is only able to liberate Fe^{3+} and Fe^{2+} from nonheme iron complexes.

Considering the above findings altogether, it is clearly evident that loosely bound nonheme Fe (II) would exist in the cytoplasm of hepatocytes, Kupffer cells, splenic macrophages, and gastric parietal cells, probably in several subcellular compartments and/or what is called the low molecular weight iron pool of the cytosol. In agreement with this view, recent studies based on quenching of the fluorescent transition metal indicator by chelatable iron and dequenching by iron chelators, demonstrated the presence of chelatable Fe (II) in the cytoplasm, nucleus, and mitochondria of isolated hepatocytes of the rat (Petrat et al. 2001, 2002). It is also possible that ubiquitous iron–sulfur proteins have iron in mixed oxidation states (Cotton et al. 1999).

Ischemia and reperfusion injure vital organs including the brain, heart, liver, kidney, and others through generation of highly toxic -OH radicals (Fenton reaction), in which Fe^{2+} dissociated in the cytoplasmic nonheme iron pool has been believed to catalyze the reaction (Freeman and Crapo 1982; Meneghini 1997). Thus, the perfusion-Turnbull + DAB method would visualize such populations of cells that are at risk from free radical damage. Furthermore, changes in nonheme iron deposition have been described in various degenerating diseases of the central nervous system (Gerlach et al. 1994). For the understanding of the mechanism of tissue and organ pathology where altered iron homeostasis has been implicated, the perfusion-Perls and -Turnbull + DAB methods may provide useful knowledge.

Since flushing the vascular system with a small volume of PBS or a small volume of formalin–PBS fixation prior to perfusion iron histochemistry did not affect the results, these procedures may be helpful for thorough perfusion and for in situ preservation of cell and tissue structures. By perfusion of the fixatives through the major arteries of organs, the perfusion-Perls and -Turnbull + DAB methods are applicable to normal and pathological human materials at autopsy.

References

Beinert H, Kennedy MC, Stout CD (1996) Aconitase as ironsulfur protein, enzyme and iron-regulatory protein. Chem Rev 96:2335–2373

Chisolm BM III, Hazen SL, Fox PL, Cathcart MK (1999) The oxidation of lipoproteins by monocytes-macrophages. J Biol Chem 274:25959–25962

Cooper CE (1999) Nitric oxide and iron proteins. Biochim Biophys Acta 1411:290–309

Cooper PJ, Iancu TC, Ward RJ, Guttridge KM, Peters TJ (1988) Quantitative analysis of immunogold labelling for ferritin in liver from control and iron-overloaded rats. Histochem J 20:499–509

Cotton FA, Wilkinson G, Murillo CA, Bochmann M (1999) Advanced inorganic chemistry, 6th edn. Wiley, New York

Crichton RR, Ward RJ (1992) Iron metabolism: new perspectives in view. Biochemistry 31:11255–11264

Cunha FQ, Assreuy J, Moncada S, Liew FY (1993) Phagocytosis and induction of nitric oxide synthase in murine macrophages. Immunology 79:408–411

Feng X-L, Usui H, Fujita T, Ichikawa T, Katagiri T, Washiyama K, Kumanishi T (1998) Postnatal developmental changes in NSE and NNE mRNA expression in the rat pineal gland: in situ hybridization histochemistry. J Pineal Res 24:108–116

Fontecave M (1998) Ribonucleotide reductase and radical reactions. Cell Mol Life Sci 54:684–695

Freeman BA, Crapo JD (1982) Biology of disease, free radicals and tissue injury. Lab Invest 47:412–426

Gerlach M, Ben-Shachar D, Riederer P, Youdim MBH (1994) Altered brain metabolism of iron as a cause of neurodegenerative diseases? J Neurochem 63:793–807

Gross SS (1995) Nitric oxide: pathophysiological mechanisms. Annu Rev Physiol 57:737–769

Meneghini R (1997) Iron homeostasis, oxidative stress, and DNA damage. Free Radic Biol Med 23:783–792

Monteiro HP, Vile GF, Winterbourn CC (1989) An iron chelator is not required for reductive iron release from ferritin by radical generating system. Free Radic Res Commun 7:33–35

Moos T, Møllgård K (1993) A sensitive post-DAB enhancement technique for demonstration of iron in the central nervous system. Histochemistry 99:471–475

Morris CM, Candy AE, Bloxham CA, Edwardson JA (1992) Histochemical distribution of non-haem iron in the human brain. Acta Anat 144:235–257

Nishimura Y (1910) Vergleichende Untersuchungen über die mikrochemische Eisenreaktion in menschilichen Leben. Zentralbl Allg Pathol Pathol Anat 21:10–18

Nyguen-Legros J, Bizot J, Bolesse M, Policani J-P (1980) "Noir de diaminobenzidine": une nouvelle méthode histochimique de révélation du fen exogéne. Histochemistry 66:239–244

O'Connell MJ, Ward RJ, Baum H, Peters TJ (1985) The role of iron in ferritin- and haemosiderin-mediated lipid peroxidation in liposomes. Biochem J 229:135–139

Okamoto K (1937) Über das Gewebseisen. Acta Scholae Med Kioto 20:413–561

Pearse AGE (1985) Inorganic constituents and foreign substances. In: Histochemistry, theoretical and applied, vol 2. Analytical technology. Churchill Livingstone, Edinburgh, pp 973–1033

Perl DP, Good PF (1992) Comparative techniques for determining cellular iron distribution in brain tissues. Ann Neurol 32:S76–S81

Petrat F, De Groot H, Rauen U (2001) Subcellular distribution of chelatable iron: a laser scanning microscopic study in isolated hepatocytes and liver endothelial cells. Biochem J 356:61–69

Petrat F, Weisheit D, Lensen M, De Groot H, Sustmann R, Rauen U (2002) Selective determination of mitochondrial chelatable iron in viable cells with a new fluorescent sensor. Biochem J 362:137–147

Pool CW, Buijs RM, Swaab DF, Boer GJ, Van Leeuwen FW (1983) On the way to a specific immunocytochemical localization. In: Cuello AC (ed) IBRO handbook series: methods in the neurosciences, vol 3. Immunohistochemistry. Wiley, Chichester, pp 1–46

Snook T (1964) Studies on the perifollicular region of the rat's spleen. Anat Rec 148:149–159

Straus W (1971) Inhibition of peroxidase by methanol and by methanol–nitroferricyanide for use in immunoperoxidase procedures. J Histochem Cytochem 19:682–688

Thomas CE, Aust SD (1986) Reductive release of iron from ferritin by cation free radicals of paraquat and other bipyridyls. J Biol Chem 261:13064–13070

Thomas CE, Morehouse LA, Aust SD (1985) Ferritin and superoxide-dependent lipid peroxidation. J Biol Chem 260:3275–3280

Yu S, Iwatsuki H, Ichinohe N, Mori F, Shoumura K (2001) 'In vivo perfusion Turnbull's reaction' for Fe (II) histochemistry in non-anoxic/non-ischemic and anoxic/ischemic cat brains. Neurosci Lett 308:79–82