Histochemical Approach of Cryobiopsy for Glycogen Distribution in Living Mouse Livers Under Fasting and Local Circulation Loss Conditions

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Abstract

Soluble proteins and glycogen particles, which are easily lost upon conventional chemical fixation, have been reported to be better preserved in paraffin-embedded sections by "cryobiopsy" combined with freeze-substitution fixation (FS). In this part, we mentioned the distribution of glycogen in living mouse livers under (1) food restriction and (2) local circulation loss conditions with periodic acid-Schiff (PAS) staining by cryobiopsy. (1) Livers of fully fed mice showed high PAS-staining intensity in the cytoplasm of all hepatocytes, showing much glycogen in all hepatocytes. However, treatment with α-amylase clarifies that hepatocytes around the central vein preserve more glycogen than the hepatocytes around the portal vein. At 6 or 12 h after fasting, PAS-staining intensity markedly decreased in restricted areas of zone I near the portal tracts. This result was the same as that of specimens gotten from individual mouse. On the other hand, glycogen distributions were different in local circulation loss condition which caused by temporarily clipping of liver tissues followed by recovery of blood circulation. (2) In the liver tissues in which blood was recirculated for 1 h after the 30-min anoxia, PAS staining was still observed in zone II and also in restricted areas of zone I far from the portal tracts. We propose that the level of consumption or production of glycogen particles could vary in zone I, depending on the distance from the portal tracts. Thus, cryobiopsy combined with FS enabled us to examine time-dependent changes in glycogen distribution in the liver tissues of living mice. This combination might be applicable to the clinical evaluation of human liver tissues.

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51.1 Introduction

Cryobiopsy enables us to repeatedly obtain multiple tissue samples from a single mouse under different experimental conditions [1, 2]. In this chapter, we focused on the distribution of glycogen in the mouse liver and introduced their consumption pattern clarified by cryobiopsy. And then how to distinguish consumption from leakage by damage was mentioned. A large amount of glycogen in the livers is an important energy source to be metabolized into blood glucose to sustain animal life [3]. Glycogen metabolism was related to not only feeding conditions but also ischemia-reperfusion

Fig. 51.1 Glycogen distribution of cryobiopsied liver tissue specimens stained with PAS. (**a**) Fully PAS-stained hepatocytes are clearly observed in the specimen without α-amylase treatment. (**b**) Hepatocytes around the central vein (CV) exhibit higher-intensity PAS staining than those around the portal tract (P) after treatment with α-amylase. (**c**) Slightly weaker PAS staining is detected around some portal tracts after 6-h fasting. (**d**) In the liver tissues of mice fasted for 12 h, PAS-staining intensity around the portal tracts is markedly decreased, but still observed in parts of zone I (*arrowheads*). *CV* central vein, *P* portal vein. Bars: 100 μm
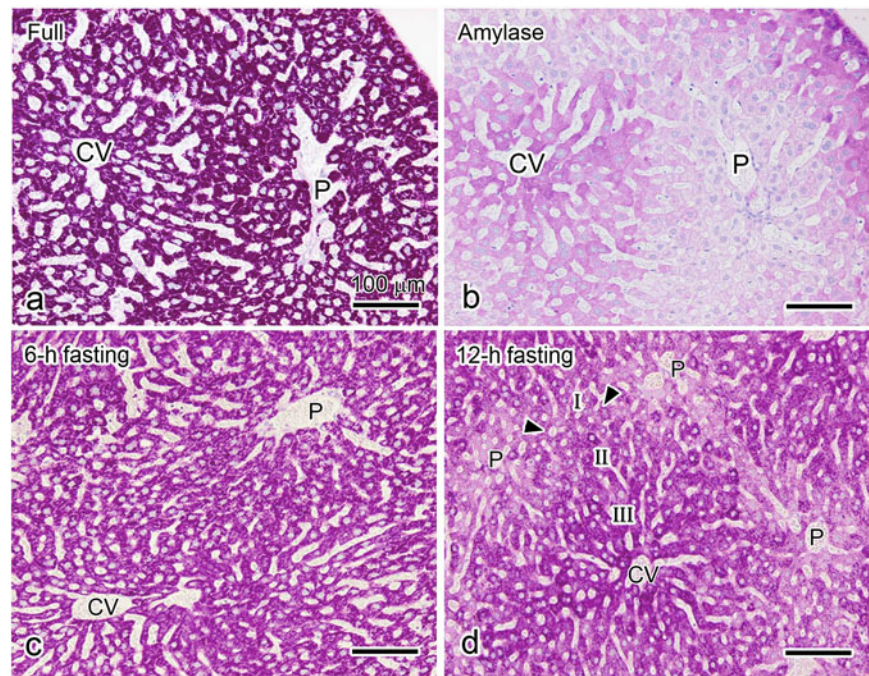
and stress of animal livers [4–8]. Ischemia and reperfusion typically occur in liver transplants and result in various types of liver damage and reactions [9–14]. Therefore, it is necessary to precisely determine time-dependent changes in the glycogen storage of hepatocytes in paraffin-embedded tissues, which reflect their living states. In conventional preparation with chemical fixatives in a typical buffer solution, the artificial loss of glycogen particles is much higher than in various cryotechniques [15].

51.2 Distinguishable Differences of a Tiny Amount of Glycogen in Hepatocytes

Cryobiopsy of liver tissues was performed on anesthetized mice [2, 16]. Briefly, after exposure of target liver lobes, a piece of tissue was quickly pinched off using handmade cryoforceps attached to sponge reservoirs of liquid nitrogen on Keisei Osteotrite Tweezers® (F-2051 or F-2052, Keisei Medical Industrial Co., Ltd., Niigata, Japan), which had already been precooled in liquid nitrogen (LN$_2$, −196 °C). The cryobiopsied liver tissues were immediately immersed in liquid isopentane-propane cryogen (−193 °C) precooled in LN$_2$ and then transferred into acetone containing 2 % paraformaldehyde at about −80 °C for starting freeze-substitution fixation (FS) [2, 16]. Cryobiopsy-FS specimen is able to choose usual paraffin embedding or sucrose embedding for frozen section. In this chapter, we're showing paraffin-embedded specimens [1]. The paraffin-embedded specimens were treated in the same way as conventional specimens. To detect glycogen in hepatocytes, deparaffinized sections were incubated in periodic acid followed by Schiff's solution and sodium metabisulfite. In mouse liver tissues of fully fed mice prepared by cryobiopsy, high-intensity PAS staining was observed as a strong red-purple color in the cytoplasm of all hepatocytes (Fig. 51.1a). However, treatment with α-amylase for serial sections digested the glycogen in hepatocytes and clarified that the hepatocytes around central veins kept much more glycogen in their cytoplasm than those around the portal veins (Fig. 51.1b). Compared with glycogen of fully fed mice, the intensity of PAS staining after fasting for 6 h became slightly weaker in hepatocytes (Fig. 51.1c). After 12-h fasting, the PAS-staining intensity had clearly decreased around almost all portal tracts (Fig. 51.1d). Most hepatocytes in parts of zone I far from the portal veins were still more PAS positive (arrowheads in Fig. 51.1d).

51.3 Repeatable Cryobiopsy for Individual Mouse

Cryobiopsy was developed for clinical and repeated collection as substitute for our "in vivo cryotechnique." To examine that the cryobiopsy was time-dependently repeatable, one piece from an area of liver tissues was obtained by cryobiopsy (Fig. 51.2a-i), and another area was cryobiopsied for 6 h (Fig. 51.2c-ii). Temporary bleeding from the tissue area pinched with the cryoforceps was quickly stopped by covering it with instant glue (Fig. 51.2b). During the continuous experiments, all the mice were warmed on an electrically
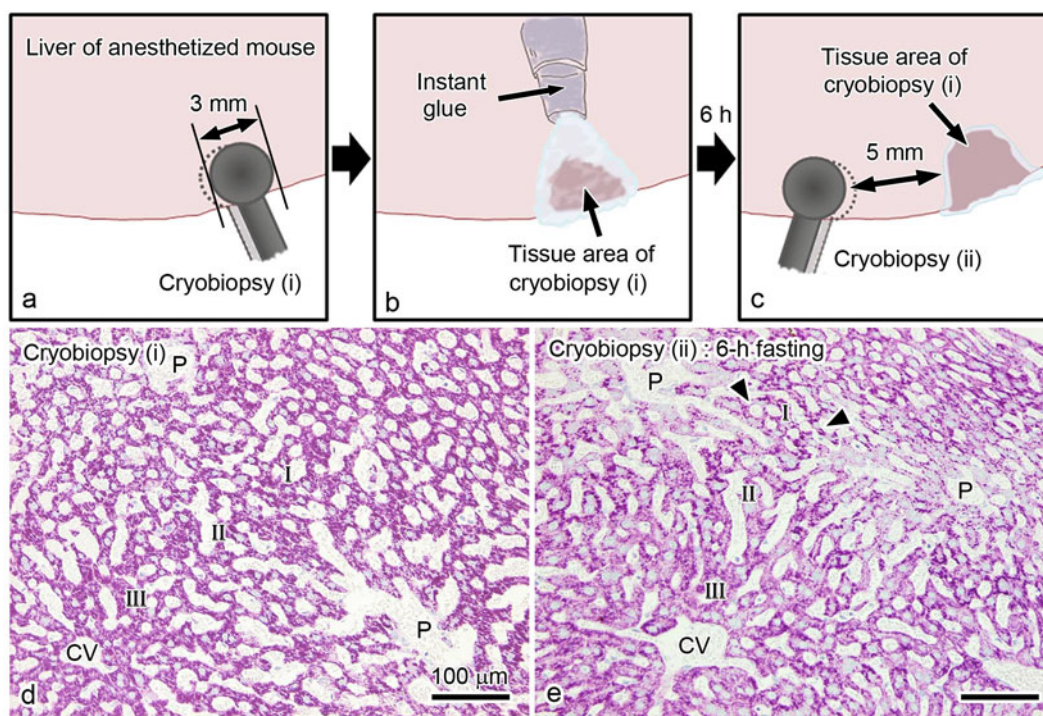
Fig. 51.2 Schematic representation of a series of cryobiopsy processes to obtain liver tissue specimens from anesthetized mice. A liver specimen is initially pinched off at one area with a pair of cryoforceps (cryobiopsy (i) in (**a**)), and bleeding is immediately stopped with an instant glue (**b**). After 6-h fasting, another liver tissue specimen is similarly obtained at a second area with the cryoforceps (cryobiopsy (ii) in (**c**)). (**d**) In the specimen of cryobiopsy (i), intense PAS staining is observed in almost all hepatocytes, the exception being those around portal veins (P). (**e**) The PAS-staining intensity of the area of cryobiopsy (ii) is found to be decreased around portal tracts. Note the strongly PAS-positive hepatocytes remaining in parts of the middle of zone I (*arrowheads*). *CV* central vein, *P* portal vein. Bars: 100 μm

heated plate to maintain their body temperature. In the liver tissues of an almost fully fed mouse, PAS staining was observed in almost all hepatocytes (Fig. 51.2a), with a slightly lower intensity in some hepatocytes around portal veins. After 6-h fasting, the PAS-staining intensity of hepatocytes had clearly decreased around the portal tracts (Fig. 51.2b), but other hepatocytes far from the portal veins in zone I were still PAS positive (arrowheads in Fig. 51.2b). Thus, the time-dependent changes in glycogen distribution can be clearly evaluated in a single mouse liver by cryobiopsy.

## 51.4 Detection of Different Consumption Under Local Circulation Loss Condition

To examine the effects of blood circulation on glycogen consumption, temporary stopping and reflowing of blood circulation were produced by binding with a circular clip and removing it (Fig. 51.3a). To prevent drying of the liver surface tissues, abdominal skin was quickly sutured, and a cotton sheet-absorbing physiological saline was placed on the mouse abdomen throughout the experiment. Temporary

hypoxia of some parts of the clipped liver tissues had already been confirmed by measuring the oxygen saturation of similar liver tissues with confocal Raman cryomicroscopy, as previously reported [17]. After stopping blood circulation for 30 min and reflowing for 1 h, cryobiopsy of the restricted areas was performed on the inside (Fig. 51.3b-I) or outside (Fig. 51.3b-II) of the clipped liver tissues. In the liver tissues inside the clipped areas, the PAS-staining intensity was markedly decreased in certain areas around the portal veins of zone I and also the central veins (zone III) (Fig. 51.3c). Even in liver tissues outside the clipped areas (Fig. 51.3c, d), the PAS-staining intensity heterogeneously decreased as a mosaic pattern in all zonal areas. Thus, the PAS staining of liver tissues by cryobiopsy clearly revealed zonal differences in glycogen storage after reflowing of blood circulation in living mice and probably reflects zonal metabolic functions in vivo.

51.5 Conclusion

The cryobiopsy presented here is a simple manual technique to capture all biological materials of living mouse livers in rapidly frozen ice crystals, the characteristics of which

Fig. 51.3 Schematic representation of the experiment about anoxic blood circulation; an area of the mouse liver tissue is clipped to stop the blood circulation for 30 min (**a**). One hour after removing the clip, some liver tissue specimens are obtained inside or outside the clipped area with cryoforceps (cryobiopsy (I) and (II) in (**b**)). Liver tissues were pinched off from inside ((**c**) cryobiopsy (I)) or outside ((**d**) cryobiopsy (II)) clipped areas, as shown in (**b**). (**c**) Marked reductions in PAS-staining intensity are detected in zone III and local areas of zone I near the portal tracts inside the clipped areas. Staining is still observed in areas far from the portal tracts in zone I (*arrowheads*). (**d**) The PAS-staining intensity is heterogeneously detected in all areas of the liver tissue outside the clipped area. *CV* central vein, *P* portal vein. Bars: 100 μm
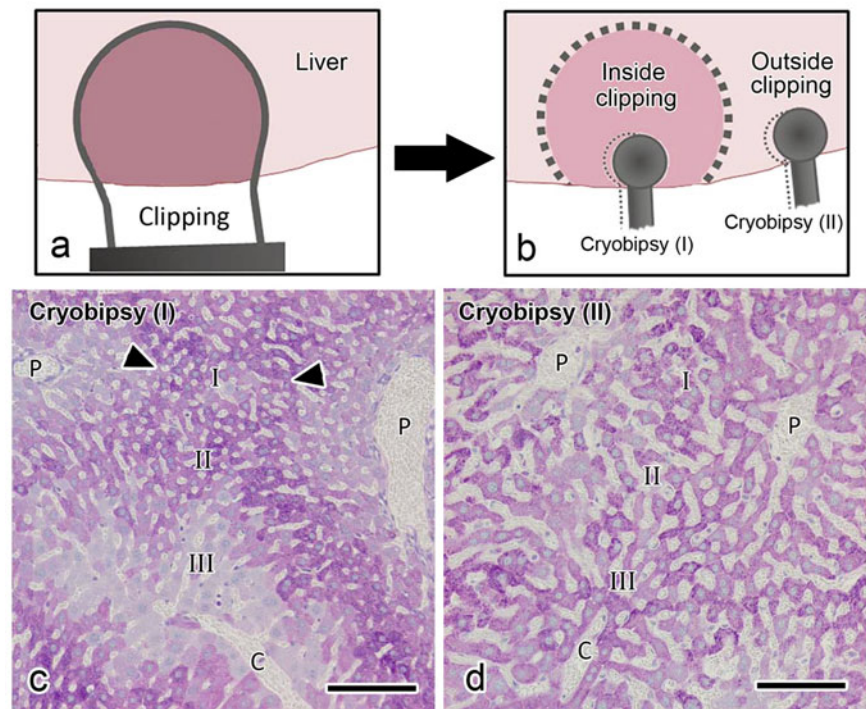
probably reflect their living states [1, 2, 16]. This does not require resection of a target organ with continuous blood circulation in vivo, results in neither ischemia nor anoxia, and enables us to obtain multiple tissue samples from a single animal and any areas of the target organ at different times. The subsequent freeze substitution in acetone with chemical fixatives is useful to preserve many glycogen particles in hepatocytes and avoids their removal during the preparation steps. Thus, the cryobiopsy technique combined with freeze-substitution fixation for PAS staining is a reliable tool to visualize changes in glycogen storage in hepatocytes of living animal livers. This text is mainly quoted from Reference [18].

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