



An Optimized Protocol for Histochemical Detection of Senescence-associated Beta-galactosidase Activity in Cryopreserved Liver Tissue

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Summary

Senescence-associated beta-galactosidase (SA- β -gal) activity assay is commonly used to evaluate the increased beta-galactosidase (β -gal) activity in senescent cells related to enhanced lysosomal activity. Although the optimal pH for β -gal is 4.0, this enzymatic activity has been most commonly investigated at a suboptimal pH by using histochemical reaction on fresh tissue material. In the current study, we optimized a SA- β -gal activity histochemistry protocol that can also be applied on cryopreserved hepatic tissue. This protocol was developed on livers obtained from control rats and after bile duct resection (BDR). A significant increase in β -gal liver activity was observed in BDR rats vs controls after 2 hr of staining at physiological pH 4.0 ($6.98 \pm 1.19\%$ of stained/total area vs 0.38 ± 0.22 ; $p < 0.01$) and after overnight staining at pH 5.8 (24.09 ± 6.88 vs 0.12 ± 0.08 ; $p < 0.01$). Although we noticed that β -gal activity staining decreased with cryopreservation time (from 4 to 12 months of storage at -80°C ; $p < 0.05$), the enhanced staining observed in BDR compared with controls remained detectable up to 12 months after cryopreservation ($p < 0.01$). In conclusion, we provide an optimized protocol for SA- β -gal activity histochemical detection at physiological pH 4.0 on long-term cryopreserved liver tissue: (**J Histochem Cytochem XX: XXX–XXX, 2020**)

Keywords

beta-galactosidase, bile duct resection, biliary cirrhosis, cryopreservation, liver tissue, senescence

Introduction

Senescence-associated beta-galactosidase (SA- β -gal) activity is a widely used biomarker of cellular senescence both in cultured cells and in tissue sections, using histochemical staining. The SA- β -gal activity results from an increased expression of *GLB-1*, the gene encoding the lysosomal beta-galactosidase (β -gal).¹ The increase in *GLB-1* mRNA and protein levels results from an elevation of the number and activity of lysosomes, which is probably due to the accumulation of damaged macromolecules in senescent cells.² Even if the optimal pH of reaction for β -gal is 4.0, the enzymatic activity has been most commonly analyzed at pH 6.0 in senescent cells, due to its increased lysosomal production related to senescent conditions. Hence, the SA- β -gal activity was originally defined as the β -gal activity detected at pH 6.0.³ Subsequently, to

increase the sensitivity of the assay, the SA- β -gal activity was sometimes measured at slightly lower pH values (5.5–5.8) according to cell type.^{4,5}

Interestingly, patients with GM1 gangliosidosis (defective β -gal) do not show any SA- β -gal activity when their cells reach replicative senescence, suggesting that the SA- β -gal activity is not itself required for cell senescence.¹ Still, the SA- β -gal activity is present in most senescent cell types, making it a very useful tool to monitor this feature. Over the

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years, this test has been used in a large variety of cell cultures and tissues in rodents and humans, and especially in human fibroblasts.^{2,4,6} Although several studies briefly mentioned SA- β -gal activity tests on liver tissue, no proper and detailed protocol is currently available. Experimental parameters including freezing and fixation methods, as well as the thickness of the slides and the pH of the solution were not consistently described (Supplementary Table 1).^{5,7–19} Cryopreservation techniques were not detailed, and the section thickness could vary between 4 and 7 μ m when mentioned. Fixation methods varied widely, with 0.2% to 0.5% glutaraldehyde and/or 3% to 4% (para)formaldehyde for 1 min to 4 hr, before or after freezing, and at room temperature (RT) or 4C. The pH of the solution ranged between 5.5 and 6.0, as suggested in other cellular types.^{4,5}

The aim of this study was to deeply optimize a protocol that measures β -gal activity and its kinetics in normal and diseased liver tissue, based on the previously documented methodologies.^{20,21} To develop and evaluate the usefulness of our protocol, we used the murine bile duct resection (BDR) model of biliary cirrhosis, in which the SA- β -gal activity has previously been described on fresh liver tissue.^{22,23} By optimizing the histochemical reaction conditions, we aimed at extending the use of the SA- β -gal activity assay to cryopreserved tissue to facilitate future research.

Materials and Methods

Rat Liver Samples and BDR

Liver tissue was obtained from wild-type male Wistar rats. Biliary cirrhosis was induced in 2-month-old rats ($n=6$) with the extrahepatic BDR surgical procedure as previously described.²⁴ Animals were sacrificed 2 or 4 weeks after the surgical procedure, and they all presented with histological biliary cirrhosis at the time of sacrifice. Controls ($n=4$) underwent sham procedure (bile duct dissection without resection) at the same age and were sacrificed at the same time points. Healthy 2-month-old male Wistar rats ($n=4$) were used for specific tests. This project was approved by the Ethical Committee for Animal Experimentation of Université Catholique de Louvain, Brussels (2018/UCL/MD/43).

β -gal Activity on Cryopreserved Liver Tissue Protocol

The final β -gal activity protocol that we developed and applied on cryopreserved liver samples is the following:

1. Briefly rinse fresh tissue with NaCl 0.9% to remove excess residual blood.
2. Immediately embed the tissue in OCT compound (Tissue-Tek OCT compound by Sakura, Torrance,

CA) and flash-freeze in liquid nitrogen containing isopentane (Fisher Scientific, Waltham, MA; CAS no. 78-78-4):

- i. Fill a metal beaker with two thirds of isopentane and place it in enough liquid nitrogen to reach about one third of the container (prepare at least 10 min before freezing).
 - ii. Freeze the sample in the clear portion of isopentane without fully submerging.
 - iii. Avoid block cracking by removing the sample from isopentane when there is still a small drop size of unfrozen OCT. Transfer sample to dry ice while continuing on to other samples.
3. Immediately section the frozen tissue in -20°C cryostat (15 μ m sections) or store the samples at -80°C for up to 12 months. The sections must remain in the cryostat chamber during the procedure to avoid tissue thawing.
 4. Fixation is done with 0.2% glutaraldehyde (Merck, Kenilworth, NJ; CAS no. 111-30-8) in PBS for 10 min at RT.
 5. Remove the fixative and wash in PBS (enough to cover the slides) for 1 min three times at RT.
 6. Add freshly prepared staining solution²: 40 mM citric acid monohydrate/sodium dihydrogen phosphate monohydrate buffer at pH 4.0 or pH 5.8 as needed (Merck, CAS no. 5949-29-1 and 10049-21-5), 5 mM potassium hexacyanoferrate (II) trihydrate (Merck, CAS no. 14459-95-1), 5 mM potassium hexacyanoferrate (III) (Merck, CAS no. 13746-66-2), 150 mM sodium chloride (Merck, CAS no. 7647-14-5), 2 mM magnesium chloride hexahydrate (Merck, CAS no. 7791-18-6), and 1 mg/ml (pH 5.8) or 0.5 mg/ml (pH 4.0) 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (Merck, CAS no. 7240-90-6) in distilled water. Incubate a control section in the absence of X-gal to verify the specificity of the enzyme reaction.
- Note: 200 μ l of solution per section is enough if a hydrophobic pen is used to surround the tissue (Dako Pen; Agilent, Santa Clara, CA). Otherwise, the slides must be fully immersed in the solution. Additional details concerning the preparation of the solutions are available in the protocol of Debacq-Chainiaux et al.²
7. Incubate overnight at 37°C without CO_2 (or shorter period for pH 4.0 manipulations); wash twice in PBS.
 8. Counterstain 1 min in hematoxylin if indicated; wash twice in PBS.

Note: counterstaining helps identifying tissue architecture, but can make a weak staining harder to detect.

Quantification of β -gal Activity Staining in Whole Tissue Sections

Stained slides were digitalized using an SCN400 slide scanner (Leica Biosystems; Wetzlar, Germany) at 20 $\times$ magnification (40 $\times$ for illustrations). Scanned slides were then analyzed using the image analysis tool Author version 2017.2 (Visiopharm; Hørsholm, Denmark) for

β -gal activity staining digital quantification. Using a first Analysis Protocol Package (APP), tissue was automatically delineated at a low digital magnification (4 $\times$) (Fig. 1A). Large empty spaces (vessels, damaged tissue) were automatically discarded. This first delineation operation was visually checked and manually corrected if required. Histological artifacts were manually discarded. Within the final selected regions, β -gal staining was detected at high resolution (20 $\times$) in a second APP with a thresholding classification relying on the RGB-R (red green blue – red) matrix of the software, which was tested and optimized manually (Fig. 1B). Results were

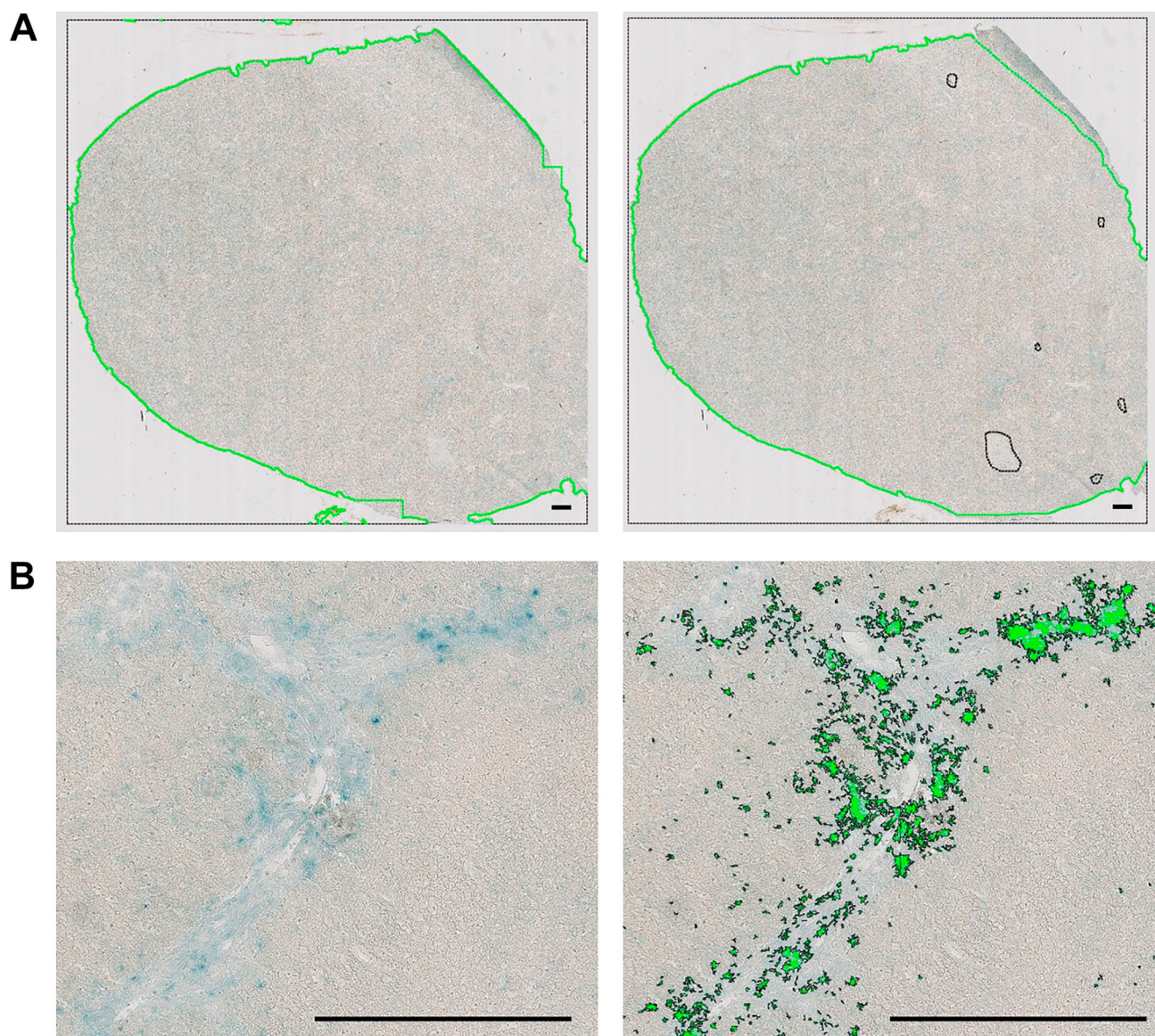


Figure 1. Quantification of beta-galactosidase (β -gal) staining with Visiopharm software. (A) The left image shows the automatic tissue delineation obtained with the first Analysis Protocol Package (APP) (green line), and the right image shows the final delineation after manual correction and exclusion of histological artifacts (surrounded by a black circle). (B) The left image shows the pathological liver tissue with histochemical β -gal activity staining before digital staining detection (positive blue staining), and the right image shows the digital β -gal staining detection obtained with the second APP (positive green staining). Scale bars = 500 μ m.

expressed as [(stained area/total tissue area) $\times$ 100] to obtain a percentage of stained area. The parameters of the designed APPs were kept constant for all sections.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 5.0 (GraphPad Software; La Jolla, CA). Continuous variables were presented as mean $\pm$ standard error of the mean. The Mann–Whitney *U*-test was used to compare continuous variables between subgroups. Student's paired *t*-test was used when specified in the text, to compare repeated measures. A two-tailed $p \leq 0.05$ was considered to indicate statistical significance for all analyses.

Results

Optimization of β -gal Activity Assay

Different reaction parameters were tested on 2-month-old healthy Wistar rat livers ($n=4$) at physiological pH 4.0 (Fig. 2). Fixation with 0.2% glutaraldehyde for 10 min at RT provided a higher β -gal staining ($9.79 \pm 2.17\%$ of stained/total area) compared with 1% formaldehyde for 1 min at RT (0.09 ± 0.04 ; $p < 0.05$) or in the absence of fixation (0.08 ± 0.04 ; $p < 0.05$) (Fig. 2A).

Increasing the substrate (X-gal) concentration from 1 mg/ml to 2 mg/ml did not enhance the intensity of β -gal staining ($9.79 \pm 2.17\%$ of stained/total area vs 2.99 ± 0.74 ; $p = 0.34$) (Fig. 2B). Furthermore, decreasing the X-gal concentration from 1 mg/ml to 0.5 mg/ml did not decrease the staining ($9.79 \pm 2.17\%$ of stained/total area vs 10.72 ± 2.59 ; $p = 0.49$). β -gal staining increased for section thickness of 10 and 15 μ m compared with 5 μ m ($9.79 \pm 2.17\%$ and $27.39 \pm 5.9\%$ of stained/total area vs 0.57 ± 0.31 ; $p < 0.05$) (Fig. 2C).

No difference in β -gal staining was noticed when we compared the following cryopreservation methods: freezing OCT-embedded specimens in liquid nitrogen containing isopentane vs snap freezing in liquid nitrogen before OCT embedding ($9.79 \pm 2.17\%$ of stained/total area vs 3.58 ± 1.83 ; $p = 0.06$). However, the isopentane method showed a better preservation of the tissue quality compared with snap freezing (Fig. 2D). Finally, although the pH 4.0 solution left overnight allowed physiological β -gal activity staining in healthy rats, β -gal staining overnight at pH 5.8 remained negative ($9.79 \pm 2.17\%$ of stained/total area vs 0.04 ± 0.01 ; $p < 0.05$) (Fig. 2E).

Altogether, the following conditions were selected to obtain an optimal β -gal staining signal for the further experiments: freezing of OCT-embedded fresh specimens in liquid nitrogen containing isopentane, slicing

the liver at 15- μ m thickness sections, and fixation with 0.2% glutaraldehyde for 10 min at RT (see "Materials and Methods" for detailed protocol). The use of 0.5 mg/ml X-gal was selected for the final β -gal activity staining protocol at pH 4.0. A reduction of the X-gal concentration was not attempted for β -gal staining at pH 5.8 because we considered this pH as already being a suboptimal reaction condition.

β -gal Activity at pH 4.0 on Cryopreserved Liver Tissue

β -gal activity staining at pH 4.0 was tested after different reaction durations in BDR (biliary cirrhosis) rats ($n=4$) vs controls ($n=2$) (Fig. 3A). Cirrhotic rats seemed to have a higher percentage of β -gal staining (% of stained/total area) at pH 4.0 compared with controls from early time points. To confirm this observation, we compared β -gal staining at pH 4.0 after 2 hr of reaction in BDR rats ($n=6$) vs controls ($n=4$). Three liver cryosections obtained after 3 to 12 months of cryopreservation were stained per rat. For each of the three staining procedures, all sections were obtained at the same cryopreservation time point. The mean of those triplicates is presented for each rat in Fig. 3B. The staining after 2 hr of reaction at pH 4.0 was higher in BDR rats vs controls (6.98 ± 2.92 vs 0.38 ± 0.22 ; $p < 0.01$) (Fig. 3B and C). When each replicate was considered separately, a significant difference in β -gal staining after 2 hr at pH 4.0 was evidenced between BDR and controls after 3, 4, and 12 months of -80°C storage ($p < 0.01$) (data not shown). All controls remained negative after 2 hr of reaction at pH 4.0 for up to 12 months after cryopreservation. The impact of cryopreservation duration was investigated by comparing β -gal staining at pH 4.0 (2 hr) between the replicates mentioned above, obtained from the same rat after different -80°C freezing intervals (Fig. 3D). β -gal activity staining (% of stained/total area) in BDR rats was lower after 12 months compared with 4 months after cryopreservation (2.23 ± 0.61 vs 7.58 ± 1.8 , $p < 0.05$; Student's paired *t*-test, $n=6$).

β -gal Activity at pH 5.8 on Cryopreserved Liver Tissue

β -gal activity staining was also tested after different times of reaction at pH 5.8 in BDR (biliary cirrhosis) rats ($n=4$) vs control samples ($n=2$) (Fig. 4A). Cirrhotic rats did not show any β -gal activity staining at early time points at pH 5.8, whereas controls did not show any activity even after overnight incubation. After 20 hr of reaction at pH 5.8, β -gal activity staining (% of

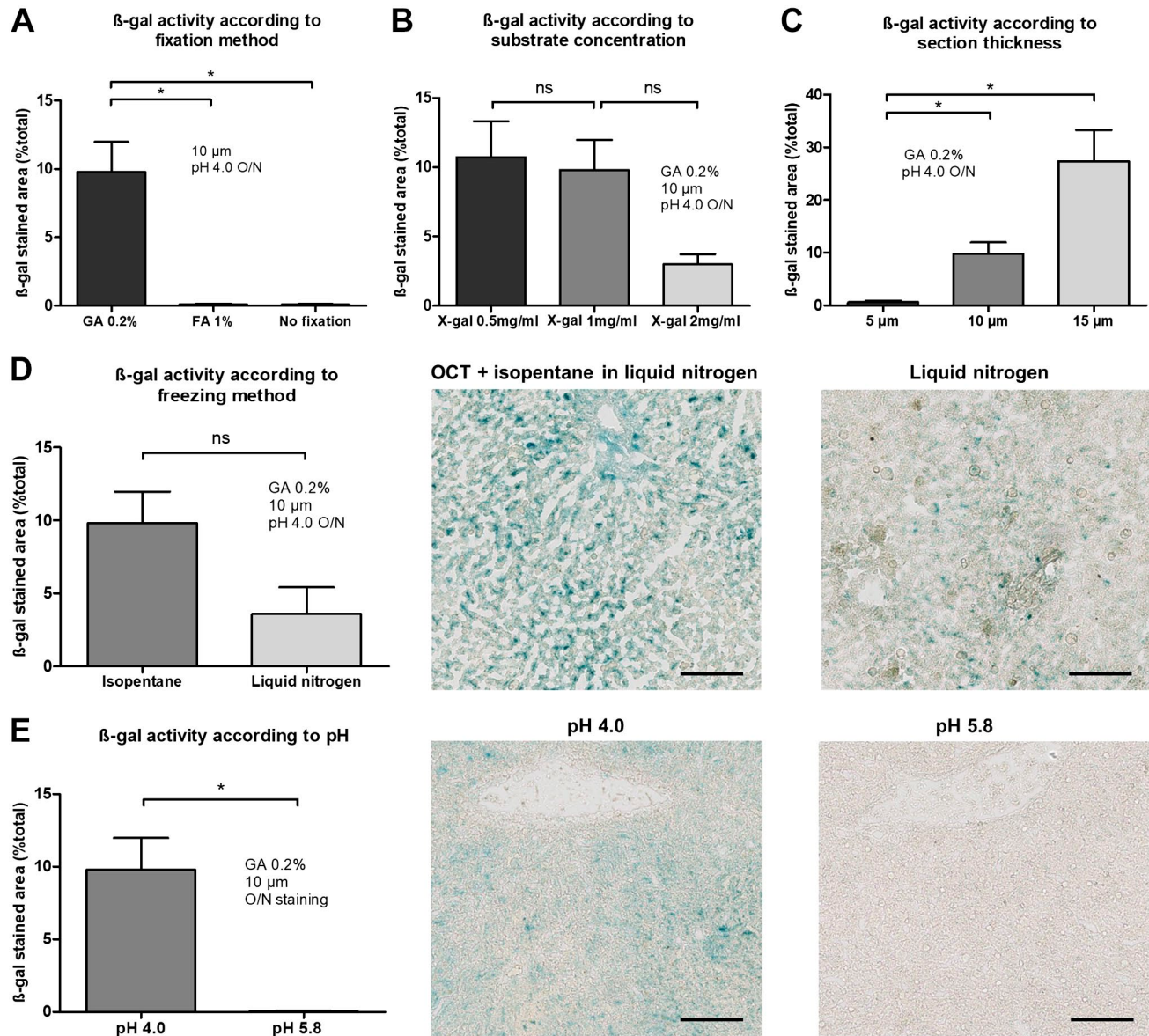


Figure 2. Optimization of experimental conditions for the detection of β -gal activity in healthy rat liver tissue. (A) Fixation with GA 0.2% for 10 min vs FA 1% for 1 min vs no fixation. (B) Usual (1 mg/ml) vs doubled (2 mg/ml) or reduced (0.5 mg/ml) X-gal concentration. (C) Section thickness of 5 μ m vs 10 μ m vs 15 μ m. (D) OCT-embedded specimens frozen in liquid nitrogen containing isopentane vs flash-freezing in liquid nitrogen followed by OCT embedding. (E) Testing physiological pH (pH 4.0) vs SA- β -gal activity pH (pH 5.8). Data are presented as mean $\pm$ standard error of the mean ($n=4$). Scale bars = 100 μ m. Abbreviations: GA, glutaraldehyde; FA, formaldehyde; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; β -gal, beta-galactosidase; O/N, overnight; SA- β -gal, senescence-associated beta-galactosidase.

stained/total area) was significantly higher in BDR rats ($n=6$) vs controls ($n=4$) (24.09 ± 6.88 vs 0.12 ± 0.08 ; $p<0.01$) (Fig. 4B and C). Three liver cryosections obtained after 4 to 12 months of cryopreservation were stained per rat. For each of the three staining procedures, all sections were obtained at the same cryopreservation time point. The mean of those triplicates is

presented for each rat in Fig. 4B. When each replicate was considered separately, a significant difference in β -gal staining after 20 hr at pH 5.8 was evidenced between BDR and controls after 4, 6, and 12 months of -80°C storage ($p<0.01$) (data not shown). All controls remained negative after 20 hr of reaction at pH 5.8 up to 12 months after cryopreservation. The impact of

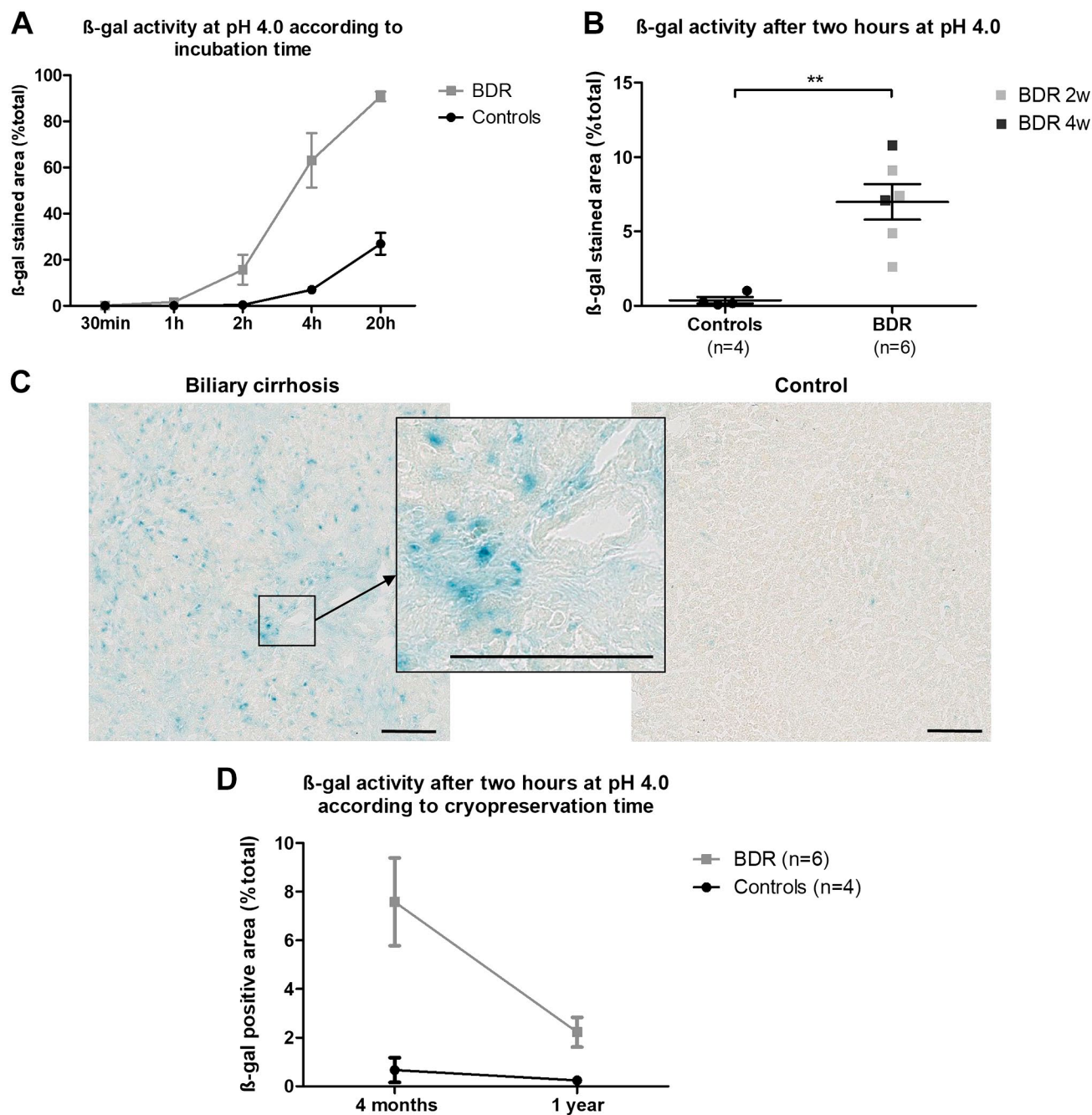


Figure 3. Analysis of β -gal activity at pH 4.0 in the livers of rats with biliary cirrhosis. (A) β -gal activity (pH 4.0) after different incubation times in rats which underwent BDR ($n=4$) (sacrificed 2 weeks [$n=2$] and 4 weeks [$n=2$] after surgery) vs controls (sham procedure, $n=2$). (B) β -gal staining comparison between biliary cirrhosis rats (BDR, $n=6$) and controls (sham procedure, $n=4$) after 2 hr of reaction at pH 4.0. Each point is the mean of three replicates for the same animal, from liver samples obtained after 3 to 12 months of cryopreservation (** $p<0.01$). (C) Images of biliary cirrhosis (BDR 2w) vs control after 2 hr of staining at pH 4.0 (liver tissue cryopreserved for 4 months). (D) Variations of β -gal activity staining after 2 hr of reaction at pH 4.0 between replicates of the same animals according to cryopreservation time. Data are presented as mean $\pm$ standard error of the mean. Scale bars = 100 μ m. Abbreviations: β -gal, beta-galactosidase; BDR, bile duct resection; BDR 2w, BDR rats sacrificed 2 weeks after surgery; BDR 4w, BDR rats sacrificed 4 weeks after surgery.

cryopreservation duration was investigated by comparing β -gal staining at pH 5.8 (overnight) between replicates obtained from the same rat after different -80°C freezing intervals (Fig. 4D). There was a decrease in

β -gal activity staining (% of stained/total area) in BDR rats 12 months after cryopreservation vs 4 months (10.22 ± 5.11 vs 34.06 ± 9.94 , $p<0.01$; Student's paired t -tests, $n=6$).

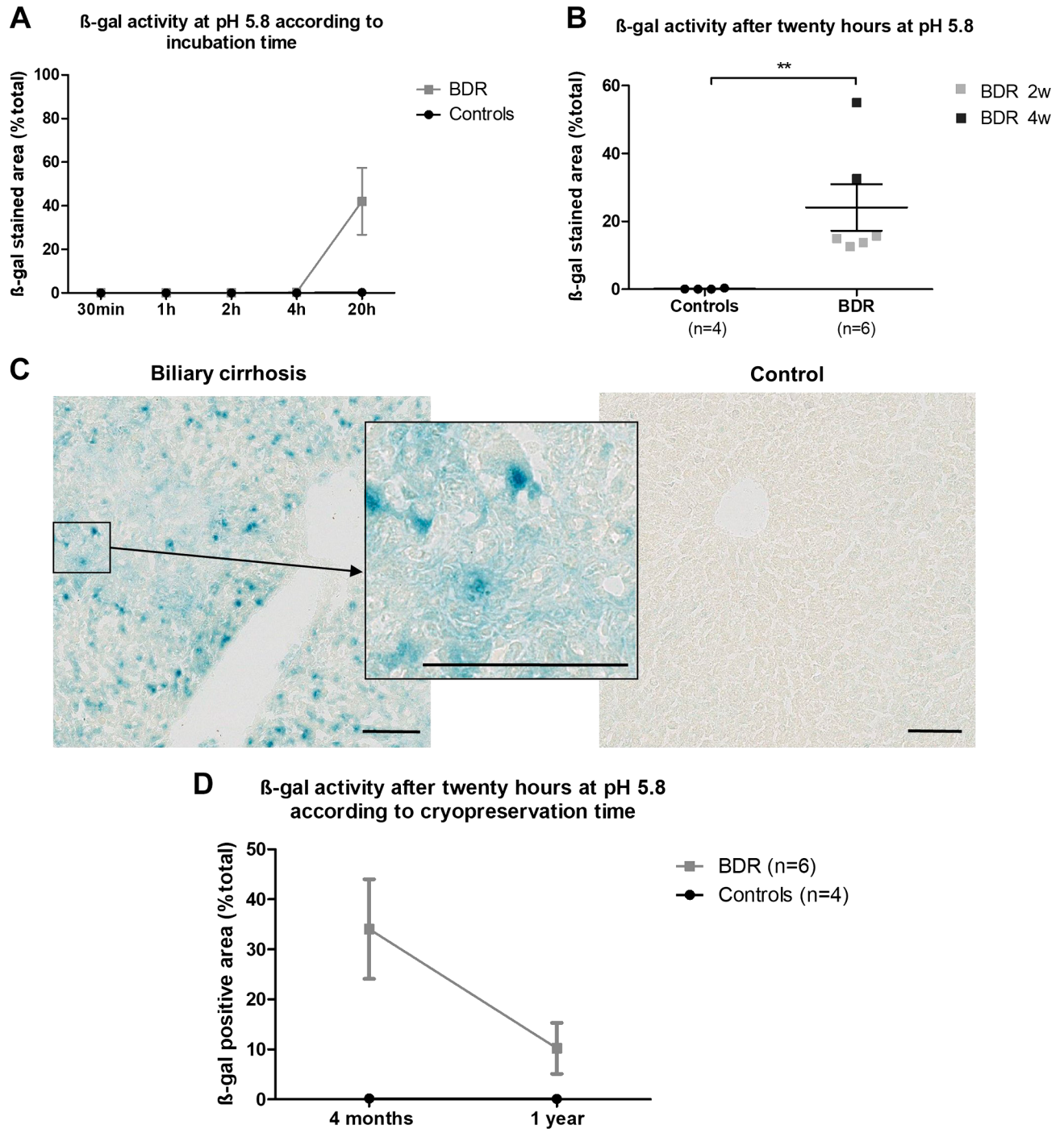


Figure 4. Analysis of β -gal activity at pH 5.8 in the livers of rats with biliary cirrhosis. (A) β -gal activity (pH 5.8) after different incubation times in rats which underwent BDR ($n=4$) (sacrificed 2 weeks [$n=2$] and 4 weeks [$n=2$] after surgery) vs controls (sham procedure, $n=2$). (B) β -gal staining comparison between biliary cirrhosis rats (BDR, $n=6$) and controls (sham procedure, $n=4$) after 20 hr of reaction at pH 5.8. Each point is the mean of three replicates for the same animal, from liver samples obtained after 4 to 12 months of cryopreservation (** $p<0.01$). (C) Images of biliary cirrhosis (BDR 2w) vs control after 20 hr of staining at pH 5.8 (liver tissue cryopreserved for 4 months). (D) Variations of β -gal activity staining after 20 hr of reaction at pH 5.8 between replicates of the same animals according to cryopreservation time. Data are presented as mean $\pm$ standard error of the mean. Scale bars = 100 μ m. Abbreviations: β -gal, beta-galactosidase; BDR, bile duct resection; BDR 2w: BDR rats sacrificed 2 weeks after surgery; BDR 4w: BDR rats sacrificed 4 weeks after surgery.

Discussion

The SA- β -gal histochemical staining protocol proposed here is a significant improvement of existing protocols. By performing the histochemical reaction at optimal assay conditions, it allows a more sensitive detection of the enzyme activity and a semiquantitative assessment of this activity in different zones of the liver tissue. It also allows to detect activity in cryopreserved tissue, which was not the case for previously reported protocols. The structural quality of tissue was better preserved when isopentane was used for freezing compared with snap freezing in liquid nitrogen, which is in line with our previous histochemistry protocols on cryopreserved human and rodent liver tissue.^{20,21,25} This was probably caused by the unpredictable freezing pattern of liquid nitrogen related to the vapor barrier, which can cause important freezing artifacts.²⁶ The fixative choice was a second crucial part of the protocol, as only glutaraldehyde allowed to preserve sufficient β -gal activity detectable via histochemical analysis. The absence of fixative probably caused a leak of the enzyme from the tissue, while β -gal activity might have been lost upon formaldehyde treatment due to protein denaturation.^{27,28} Indeed, it is known that enzymes react differently to fixation conditions.²⁹ The β -gal staining increased with sections of thickness superior to 5 μ m, as expected with higher enzyme content. We did not test sections thicker than 15 μ m because a delay in substrate penetration can be observed for those sections.³⁰ A pH 4.0 solution used overnight allowed physiological staining of healthy liver tissue (positive control). No staining was observed at pH 5.8 overnight in healthy 2-month-old Wistar rats, confirming that a pH slightly lower than 6.0 can improve sensitivity without impairing specificity compared with pH 6.0. Increasing the substrate (X-gal) concentration did not improve the intensity of β -gal staining, probably because of a saturation of the reaction sites. At the optimal pH 4.0 of reaction, it was even possible to lower down X-gal concentration without impairing the β -gal staining intensity.

The study of β -gal kinetics at pH 4.0 allowed us to evidence a noticeable SA- β -gal activity staining at early times of incubation as seen in rats with biliary cirrhosis (BDR model). A significant difference in β -gal staining between BDR rats and controls was already revealed after 2 hr of pH 4.0 reaction. Conversely, SA- β -gal activity at early times of incubation was nearly undetectable at pH 5.8 in BDR rats. After overnight incubation at pH 5.8, there was a significant difference in β -gal activity staining between BDR and control rats. Our results show that SA- β -gal activity can be evidenced at early time points at pH 4.0, as well as at late time points at pH 5.8. Close monitoring of the

negative controls is mandatory at pH 4.0, because all tissues will eventually stain positive for β -gal activity due to low concentration of the enzyme also in normal tissue. Still, senescent tissues have earlier and more intense staining at pH 4.0 compared with non-senescent ones. The state of the art in histochemistry is to test enzymatic activity at the optimum pH of the enzyme because even small variations of pH can drastically affect the results.^{20,31} On this basis, optimizing SA- β -gal activity assay to pH 4.0 should allow the detection of senescence at earlier stages compared with the suboptimal pH 5.8. Further demonstration on mildly senescent tissues will have to be conducted to confirm this hypothesis. Identifying the type of liver senescent cells with specific markers should contribute to a better understanding of the BDR model in the future.

Enzyme activity can be affected by cryopreservation to a different extent according to the type of enzyme and storage conditions.³² When we investigated the impact of -80°C cryopreservation on our liver tissue samples, it appeared that the β -gal staining tended to decrease with freezing time. This result might be related to the loss of the enzyme activity caused by cryopreservation.³² However, a significant difference in β -gal staining between BDR and controls was still observed 12 months after cryopreservation. Cryopreservation can also potentially lead to an increase in the enzyme activity detected, due to lysosomal membrane leakage caused by cryopreservation.³² Still, all controls remained negative at pH 4.0 (2 hr) or pH 5.8 (overnight) for up to 12 months after cryopreservation. According to our results, the SA- β -gal activity assay remains a specific test despite several months of tissue cryopreservation, as long as all specimens (senescent tissues and controls) are analyzed after the same storage duration. This observation is of major importance as no study so far has evaluated the impact of cryopreservation on β -gal activity staining. Being able to use cryopreserved tissue allows multiple testing on the same samples and should facilitate further research involving SA- β -gal activity assay. However, we observed a noticeable interexperimental variability, which could be explained by a variability in section location and/or by a difference in tissue senescence intensity between animals. Due to the interexperimental variations that we observed, negative controls are mandatory to corroborate results on cryopreserved tissue.

In conclusion, our results provide the first detailed optimized protocol for SA- β -gal activity staining on rat liver tissue cryopreserved for up to 12 months. We demonstrated that SA- β -gal activity can be evidenced in cryopreserved tissue after 2 hr of reaction at physiological pH 4.0, as well as after overnight staining at pH 5.8.

Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

EMS, AD, and MN conceptualized the research project and reviewed the manuscript; MR and GJ operated the animals; and GJ performed the β -gal activity tests, processed the results, and drafted the manuscript. All authors read and approved the final manuscript.

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