X-gal staining of canine skin tissues: A technique with multiple possible applications

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Abstract

Background: Estimation of β-galactosidase (βgal) activity in human cells and tissues indicate its possible use as a marker of senescence. **Objectives:** This study was done to detect senescence-associated βgal (SA-βgal) activity in canine skin tissue by using its substrate 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal). **Materials and Methods:** Skin samples were collected through rapid necropsy process. The X-gal staining was done by altering different factors of the staining procedure like pH of the reagents and incubation time. Further, effect of tissue preservation procedure was also evaluated. **Results:** Typical X-gal staining was detected in old dogs' skin samples and it was detectable both at pH 6 and pH 7.3. The cells present in the inner lining of the hair follicles and sebaceous glands are the major cells that have high SA-βgal activity. The X-gal staining intensity was more prominent in tissues preserved in liquid nitrogen at -196°C than in -80°C freezer. Prolonged incubation period increased the intensity of staining. **Conclusions:** This study indicates possibility of X-gal staining in canine tissues and opens an avenue for further in-depth studies that might be useful for different research and clinical studies like determination of dog's approximate age.

Key words: β-galactosidase, canine, senescence, skin, X-gal

INTRODUCTION

There is no doubt that dog is the most favorite pet animal throughout the world. Further, due to its physiological similarity with human being, it has turned out to be an ideal model for studying human diseases like diabetes and cancer.[1] Therefore, a better understanding about the normal physiological events of dogs might have paramount significance for clinicians, researchers, and pet owners. Physiology of an animal or human is age-dependent. However, in many cases it is very difficult to have an accurate age estimation of a dog. This is because: (i) Dog owners fail to keep the birth record of their pets, and it is not possible in stray dogs, (ii) dogs like any other companion animal is sold and resold more than once during their lifetime; hence, change of ownership may cause loss of data regarding age, (iii) normal available techniques are not very accurate and/or affordable, and (iv) very less information is available about age-related biomarkers in dogs. In a dog, denture wear, loss of elasticity of skin, rough hair coat, and so on are the common external appearances in old age. During this period, hematological alterations like anemia, lowered PCV (Packed Cell Volume), and so on and biochemical changes like decreased serum protein, increased cholesterol, and BUN (Blood Urea Nitrogen) level, and so on are evident which can be correlated with dysfunction of different organs like liver, kidney, heart, spleen, nervous, and musculoskeletal system, and so on. Many disease conditions and/or diseases are also common in geriatric dogs like glaucoma, arthritis, skin disease, arteriosclerosis, prostrate hyperplasia, brown pigmentation of internal organs, and so

on. However, all these age-related alterations are not very specific for age determination. Importantly, most of these only indicate about the presence or absence of old age. They do not give an idea about the intermediate stages of age progression from young to old age. Therefore, efforts should be made to identify novel age-related biomarkers.

In many organs, somatic cells undergo a state of permanent growth arrest and altered function after a finite number of divisions, and this is known as replicative senescence.[2] The process of replicative senescence was being established by using the human cells, which showed limited number of doublings before arrest of cell cycle.[2,3] Senescent cells are resistant to apoptosis for a long period of time but are metabolically active. There is also evidence that senescent cells can accumulate in renewable tissues with age and at sites showing age-related pathologies like osteoarthritis and atherosclerosis. By only looking at cell morphology, senescent cells cannot be distinguished from quiescent or terminally differentiated cells in tissues. An enzyme known as β-galactosidase (βgal) is overexpressed by senescent cells.[4] Various studies have confirmed that senescent-associated βgal (SA-βgal) is same as normal lysosomal βgal.[3,4] However; in senescence, overproduction of this protein increases the overall activity of this enzyme. The normal lysosomal βgal is active at pH 4, but SA-βgal activity can be easily detected at pH 6.[5] This might be due to presence of high concentration of SA-βgal enzymes in senescent cells. On the basis of this observation that at a higher pH, SA-βgal activity can be detected, various protocols have been adapted to detect SA-βgal activity in tissues and cell cultures.[3,5,6] Particularly, the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal) has been extensively used to detect the SA-βgal activity *in vivo*. βgal cleaves X-gal and produce an insoluble blue compound, which is detectable *in situ*. This staining is well-known as X-gal staining.

The X-gal staining is a very useful assay to design a reliable protocol for the identification of senescent cells quickly with simple sectioning and staining procedure. Previous study has successfully detected SA-βgal activity in human skin biopsies.[5] Importantly, an increase in SA-βgal activity detected in human skin tissues correlated with the old age. This information encouraged us to detect SA-βgal activity in canine skin tissues. We were able to detect SA-βgal activity successfully in skin samples obtained through rapid necropsy of dead dogs.

MATERIALS AND METHODS

Reagents and solutions
Gluteraldehyde, 50% solution, reagent grade and magnesium chloride; anhydrous; high purity grade was obtained from AMRESCO (Solon, Ohio, USA). Potassium ferrocyanide (extrapure analytical reagent), potassium ferricyanide, and EGTA (Ethylene glycol tetraacetic acid) were obtained from Sisco Research Lab Pvt Ltd (Mumbai, India). Nonidet P40 Substitute was obtained from Sigma Life Science (USA). The fixative solution was prepared with gluteraldehyde (0.50%), EGTA (1.25 mM; pH 6 or 7.3), $MgCl_2$ (2.00 mM) and 1 × phosphate buffered saline (PBS) (pH 6 or 7.3). The wash buffer was made up with $MgCl_2$ (2 mM), NP-40 (0.02%) and 1 × PBS. And the X-gal stain was prepared with X-gal (0.6 mg/mL), K ferrocyanide (4 mM), K ferricyanide (4 mM), and wash buffer. All the reagents were diluted from their corresponding stock solutions.

Procurement and preservation of tissue sample
All the tissue samples used in this study were collected during necropsy of dogs that have died just 10-15 min before the necropsy procedure. Particularly, we collected sample from those dogs whose exact birth record was available with the owner. We also restricted us in collecting and using samples from dogs that have died not due to any chronic disease. Most of the samples were collected from dogs' that encountered accident and presented at the College of Veterinary Science and Animal Husbandry, Orissa for treatment, but later on succumbed to death. These cases were referred to the department of veterinary pathology for postmortem/necropsy. In this way, we were able to collect samples from three dogs (two old and one young). The samples were collected from different breeds of dogs. The necropsy was conducted at the department of veterinary pathology. Further tissue storage and processing was done at the Institute of Life Sciences, Bhubaneswar. As samples were collected from dead animals and used in a nonprofitable research study, we did not seek any animal ethical committee approval. However, before collecting the samples, we took the prior consent of the corresponding owners. Previous studies have shown that storage of snap frozen tissues might reduce the activity of SA-βgal activity. Therefore, the snap frozen tissues were processed on the same day for the X-gal staining. Out of curiosity, we also kept one fragment of the snap frozen tissue in -80°C and 7 days later processed for further sectioning and staining as described below.

Cryosectioning and staining
Tissues were taken out of liquid nitrogen or -80°C and immediately embedded in tissue freezing medium (Leica Microsystems Nussloch GmbH). Tissue sections of 7-15 micron thickness were made by using cryomicrotome (Leica CM1850). After sectioning, tissues were fixed over the charged slides. Until beginning of the staining procedure, slides with the tissue were kept on ice. For the X-gal staining procedure all the

working solutions were made fresh from corresponding stocks. Initially, slides were placed in fixative for 30 min at 4°C. Then slides were rinsed with wash buffer for 4 × 5 min. This was followed by incubation of slides with X-gal staining solution for more than 12 h in 37°C incubator. The staining container was impermeable to normal light. Two beakers containing water were kept inside the incubator for maintaining the humidity and protecting from drying of staining solution (X-gal). After overnight staining, slides were washed with PBS 3 × 5 min. At last, slides were mounted with aqueous mounting solution (VectaMount™AQ; Vector laboratories, Inc.; Burlingam, CA, USA). A consecutive frozen slide was stained using H and E. All slides were observed under Leica DM500 light microscope and representative photographs were taken.

RESULTS AND DISCUSSIONS

As discussed above, the key to distinguish a lysosomal βgal activity from SA-βgal activity is staining at different pH-range (4 or more than 6). In the current study, we noticed X-gal staining both at pH 6 and pH 7.3. As reported earlier, we observed a reduction of SA-βgal activity after freezing of tissues in -80°C. However, even after 5-6 days of storage in -80°C, we were able to detect X-gal staining; though of lower intensity. This observation is not in accordance with a previously reported statement

that "even overnight storage of tissue samples at -80°C can destroy the enzyme activity."[3] The staining was detectable after minimum of 10 h incubation and staining intensity went on increasing with an increase in incubation time. Staining was mostly noticed at the inner epithelial layers of hair follicles and sebaceous glands [Figure 1a and b]. Further, the X-gal staining intensity was more intense at pH 6.0 than pH 7.3 [Figure 2a and b]. Overall, the number of hair follicles positive for X-gal was more at pH 6.0 than pH 7.3. This indicates presence of SA-βgal activity in old age canine tissues. We did not have enough samples to make a comparison of X-gal positive cells (%) in young and old age animals; however, in the tissue of only one young dog available with us, we did not see any staining even after 20 h of incubation (data not shown).

Knowledge about the age of a dog might have multiple uses in clinic and research. Currently, available techniques for the detection of a dog's age are not very reliable. The measurement of βgal activity has been proposed to be a marker for the estimation of a human age. However, not much effort has been made to detect the expression of this protein in other species like dog. To best of our knowledge, the current study is the first report regarding possible detection of endogenous βgal activity in dog skin tissue. Detection of βgal activity in skin tissues obtained through rapid necropsy showed the feasibility of detecting this molecule's activity from just died and most potentially from live animal's skin. Further, detection of X-gal staining
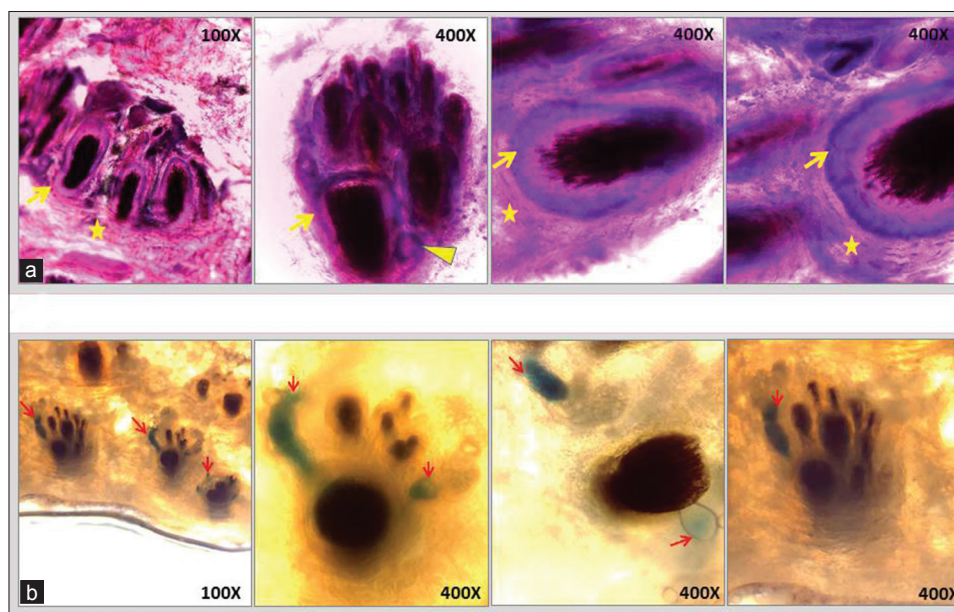
Figure 1: Histology of a dog skin tissue stained with H and E or X-gal (a) H and E stained dog skin tissue shows presence of various cellular components of epidermis and dermis. Arrows (yellow) indicate follicular epithelia cells (compound follicle), stars indicate stromal cells, and triangle indicates epithelial cells of a sebaceous gland. For H and E staining, frozen skin sample of a Greatdane dog (~5 years old) was cryosectioned and processed by usual H and E staining procedure. (b) X-gal stain of a corresponding tissue shows clear X-gal staining (red arrow) in the region of follicular epithelium and sebaceous gland. The tissue was from the same dog and processed for X-gal staining at pH 7.3. Note: Tissue used in this experiment was preserved at -80°C for 5 days after its snap freezing in liquid nitrogen
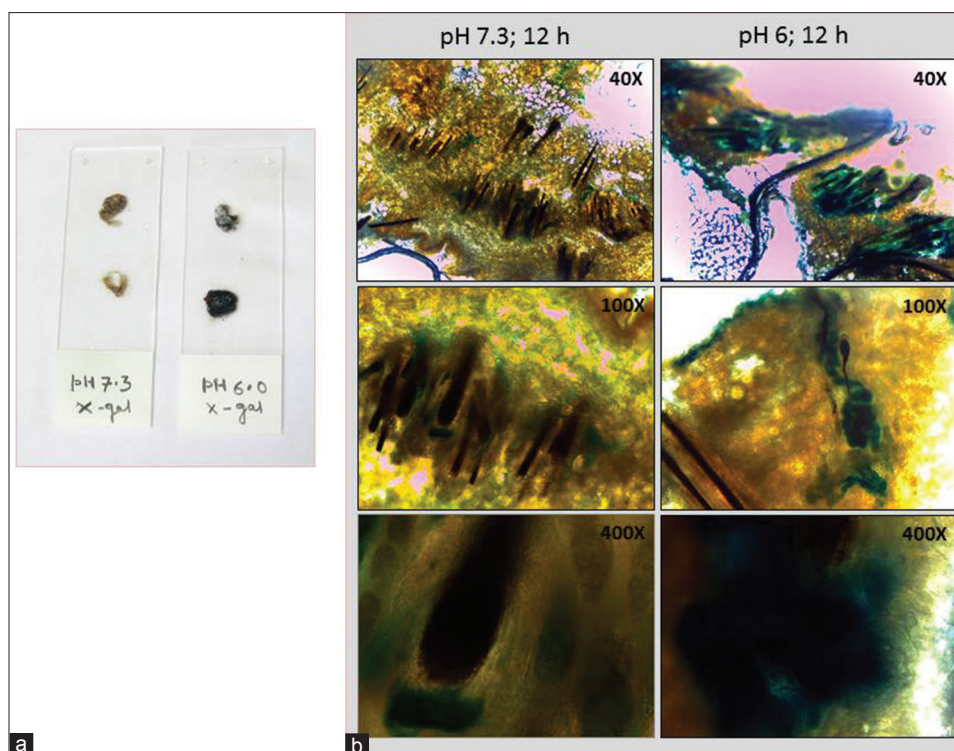
Figure 2: X-gal staining at different pH (a) Gross appearance of canine skin tissues stained with X-gal. All the staining regents were adjusted to pH 6 or 7.3. Cryosectioned skin tissues were stained for X-gal at different pH, and after overnight (12 h) staining, photos of those slides were taken by normal digital camera. Staining at pH 6 produced more intense color (blue) than at pH 7.3. (b) Microscopic images of tissues stained at different pH of staining solutions. More intense X-gal staining (blue) was observed at pH 6 than pH 7.3

efficiently in the epidermis of skin suggests requirement of a small amount of superficial skin for carrying out this analysis.

X-gal staining of canine tissues might be instrumental in estimating the proper age of a dog. This will be further useful in better treatment and management of these animals. It has also potential use in forensic science including wildlife forensic, where age estimation is warranted. For better use of this technique, an extensive analysis is required to get the actual idea about SAβ-gal activity in different age, sex, and breed of dogs. We also believe that skin tissues from different regions of the same animal might have different number of senescent cells; therefore, a more in-depth study is required before the adaptation of X-gal staining as a marker for old age of dogs. Lac-Z, the bacterial βgal, has been widely used as a reporter gene in many animal models including dogs. In most of the gene therapy studies in dog, Lac-Z has been commonly used as a reporter gene.[6,7] Lac-Z has a similarity with endogenous βgal of other species; therefore, an idea about expression level of endogenous βgal expression/activity in different organs of this animal, might help to carry out X-gal staining in a more careful fashion and proper interpretation of the results. In various literatures, most of the protocols recommend pH 7.3/7.4 for Lac-Z staining; however, at this pH we were also able to detect endogenous β-gal activity. Therefore, during Lac-Z staining of canine

tissues, tissue from the control animals should be used to avoid confusion of background staining. Furthermore, detection of senescent cells through X-gal staining in canine tissues will help in checking the coexistence and functional significance of these cells in different chronic diseases like cancer. Taken together, we believe that our current report has shown the possibility of carrying out X-gal staining with canine tissues and has created an opportunity to exploit this technique for several clinical and research studies.

CONCLUSION

On the basis of the current study, following conclusions are made:
- SA-βgal activity in dog is detectable through X-gal staining *in situ;*
- Use of X-gal staining reagents with higher pH (7.3) might help in detecting more specifically senescent cells than previously suggested pH (6);
- X-gal staining is possible with tissues collected immediately after death;
- Storage of tissues at -80°C reduce SA-βgal activity; however, X-gal staining is still feasible in those frozen tissues;
- X-gal staining of dog skin tissue has the potential to be explored as a marker of senescence or old age.

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