Journal of Neuroscience Methods 187 (2010) 59–66

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

A novel decalcification method for adult rodent bone for histological analysis of peripheral–central nervous system connections

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ARTICLE INFO

Article history:
Received 23 July 2009
Received in revised form 1 December 2009
Accepted 21 December 2009

Keywords:
Decalcification
6% TCA
Immunohistochemistry
Cryosectioning
Spinal cord
Myelin basic protein
Antigenic integrity

ABSTRACT

Histological analysis of bone encased tissue is severely hampered by technical difficulties associated with sectioning calcified tissue. Cryosectioning of bone is possible but requires significant technical adaptation and expensive materials and is often time-consuming. Some decalcifying reagents in common use result in successful cryosectioning in less time but the integrity of the soft tissue of the spinal column is often compromised during processing. This can result in significant loss of cellular detail. In order to find a method that would allow cryosectioning of the bone without loss of structural integrity of the underlying soft tissue we assessed the efficacy of four different decalcifying reagents with respect to their effects on the cellular structure of the myelin of the grey and white matter of the spinal cord. The antigenic integrity of the spinal cord white matter was evaluated using tissue structural integrity and quality of myelin basic protein immunostaining. The result of this research shows that 6% TCA not only decalcifies intact spinal column suitably for cryosectioning but does so without compromising the antigenic integrity of the tissue. The ease of application, speed of processing and a favorable cost-effective profile were secondary benefits noted with the use of the 6% TCA decalcifying solution. The ability to utilize a decalcifying solution that allows for both histomorphometry and immunohistochemistry in the same spinal column segment represents a novel technique that will provide new insights into pathophysiological aspects and therapeutic approaches ispinal cord damage or disease.

1. Introduction

There are numerous animal models of central nervous system (CNS) disorders used by researchers worldwide. (Ma et al., 2001; Frost et al., 2003; Hallam et al., 2004; Moon and Bunge, 2005; Neal et al., 2007; Durukan et al., 2008; Strbian et al., 2008; Mannie et al., 2009). A common feature of these animal models is the examination of the cellular and molecular signaling events underlying CNS disorders. Immunohistochemical examination of protein expression is frequently used for the analysis of spinal cord and brain tissue from animals of varying maturity. One of the greatest challenges to the successful completion of these studies is the ability to analyze the soft CNS tissue contained within the spinal column or cranium. In particular, the ability to analyze changes in myelin represents a critical aspect of many studies examining nervous tissue disease and damage. Myelin is a dynamic structure that wraps neuronal axons, facilitating normal nerve signal conduction, as well as maintaining the homeostasis of the peri-axonal space (Mikoshiba et al., 1991; McLaurin and Yong, 1995; Dyer, 2002). The structural integrity of the myelin sheath is critical for normal neuronal function and can be used as a marker of neuronal damage (Kurz et al., 2003; Vercellino et al., 2009). In addition to myelin analysis, the ability to examine cell signaling from the peripheral to the central nervous systems via intact connections between dorsal and ventral root systems offers a distinct advantage to explore mRNA and protein transport.

Removal of brain and spinal cord tissue from the spinal column or cranium may cause damage that compromises the accurate analysis of tissue damage resulting from the injury or disease model. In addition, removal of the tissue from the bone breaks the delicate physical connections between the peripheral and central nervous systems in the region of the spinal roots and the dorsal root ganglia connections. Maintaining the integrity of the intact connections between the peripheral and central nervous systems is essential to the advanced understanding of the bi-directional cell signalling mechanisms that are involved in the development of disorders such as MS and neuropathic pain (Altar and DiStefano, 1998; Shubayev and Myers, 2002; Ng et al., 2007). Consequently, it is preferable to cryosection the tissue while it is still contained within the bone.

Investigation of the literature revealed several different techniques available for cryosectioning of bone tissue (McElroy et al., 1993; Kawamoto, 2003; Melanson et al., 2009). These techniques involve expensive tungsten carbide cryoblades, labor intensive

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doi:10.1016/j.jneumeth.2009.12.013

techniques using special tapes and glues to obtain usable sections (Tadokoro et al., 2006), or expensive modifications of a standard cryostat (Salie et al., 2008). Alternative techniques have used plastic embedding material such as methyl methacrylate (MMA) (Gomes et al., 2008). However, the extremely high temperatures required for plastic embedding of tissue frequently destroys the antigenicity of the very proteins under investigation (Gomes et al., 2008). As a result, many researchers have preferred to decalcify the bone prior to sectioning the tissue.

The calcium in bone is mainly in the form of insoluble salts. In order to successfully cryosection the bone, the insoluble calcium–phosphate crystals need to be solubilized. This can be achieved relatively easily using dilute strong acids (such as nitric or hydrochloric), chelating agents (such as EDTA), or weak acids (such as formic, acetic or picric acids). Removal of the calcium salts leaves a fibrous collagenous structure which is easily sectioned. At present, there are several reagents available for use to decalcify bone to facilitate sectioning using a regular cryoblade. Commercial reagents include RDO-Gold and Krajian's Solution. RDO-Gold is a buffered solution of hydrochloric acid (<10%), which was formulated to facilitate rapid decalcification of bone tissue for histological analysis. Krajian's Solution, a modification of a decalcification technique first described in 1953 (Case, 1953), is a solution of formic acid and sodium citrate dihydrate. Other widely used decalcification solutions include EDTA-Glycerol solution (Mori et al., 1988; Bourque et al., 1993) or trichloroacetic acid (Graner et al., 1995a,b; Yamamoto-Fukuda et al., 2000).

Most solutions used for decalcification allow accurate histological analysis of the neuronal tissue. However, nuclear antigens including Ki-67, that are commonly used in the analysis of CNS tissue, are markedly reduced after acid decalcification protocols (Yamamoto-Fukuda et al., 2000). Further, decalcification often destroys tissue antigenicity (Bianco et al., 1988), preventing accurate analysis of molecular expression in the CNS tissue. In our study, the structure of the myelin was significantly damaged by the commercially available decalcifying reagent RDO-Gold. Therefore, we set out to determine which of the available decalcification techniques was the most suitable for the analysis of CNS tissue. We used tissue obtained from a rodent experimental autoimmune encephalomyelitis (EAE) model of MS.

After decalcification, the spinal columns were cryosectioned at 10 μm and stained using hematoxylin and eosin for gross tissue morphology, and immunostained for MBP to assess the integrity of the myelin. In addition, we performed *in situ hybridization* on the tissue to ensure the mRNA was not damaged during processing. Further, we compared the time and cost of each reagent, in order to maximize resources. We found that of the four solutions tested, decalcification with trichloroacetic acid resulted in the best spinal cord integrity as judged by histological analysis of myelin structure and stability of the mRNA. In addition, the manpower and resources used for this method are the most cost-effective of the four solutions assessed.

2. Materials and methods

2.1. Tissue preparation

Female Sprague Dawley rats were deeply anaesthetized using an I.P. injection of Ketamine (Biospacific Emeryville, CA: cat. #: A52310) 30 mg/100 g and xylazine (Bayer Health Care, Toronto, Ontario) 3 mg/100 g body weight diluted in saline. Full body perfusion was performed via intra-cardiac canulation using a pre-fixative solution containing 1U/ml heparin (LEO Pharma Inc., Thornhill, Ontario: DIN 00453811) and 1% sodium nitrate (ThermoFisher Scientific, Ottawa, Ontario: cat. # S343) in 0.9% sodium chloride (Sigma–Aldrich, Oakville, Ontario: cat. # S9625) at a volume equal to 1/3 of the animal's body weight. The animals were then perfused with a 4% paraformaldehyde (Sigma–Aldrich: cat. # 158127) in 0.1% $NaPO_4$ (ThermoFisher Scientific: cat. # S373) fixative buffer at a volume equal to 2× body weight of the animal. The whole vertebral column was removed, dissected free of surrounding soft tissue, and fixed in 4% paraformaldehyde for 24 h at 4 °C. All animal experiments were conducted in accordance with the University of Manitoba Animal Users and Protocol Management Review Committee protocols, which comply with the Canadian Council on Animal Care guidelines.

2.2. Decalcification

2.2.1. RDO-Gold (RDO-G) (less than 10% HCL)

After fixation, the vertebral column was washed in distilled water and divided into segments (<1 cm in each piece), according to the manufacturer's supplied specifications. This is to ensure that there is adequate perfusion of the solution throughout the specimen. The specimen was then placed into 6 ml RDO-Gold solution (Apex Engineering Products Corporation, Plainfield, IL). The progression of decalcification was checked with insertion of a sharp needle, as well as by chemical testing of the RDO-G solution every 30 min after the first 2.5 h (sees below). The RDO-G solution was replaced each time. After decalcification in RDO-G, the specimen was rinsed in distilled water to remove excess RDO.

2.2.2. Krajian decalcifying solution

100 g/l sodium citrate, dihydrate, and 250 ml/l formic acid (88%) in water.

After fixation, the specimen was washed in PBS for 20 min. The whole vertebral column was then decalcified in Krajian solution (Mallinckrodt Baker, Phillipsburg New Jersey: cat. # G161-02) for 4 days at 4 °C. Incubation time was assessed at several time points using insertion of a sharp needle into the bone, and chemical testing of the Krajian solution, to determine the end-point of decalcification. After decalcification, the specimen was washed in PBS for 20 min prior to processing for sectioning.

2.2.3. EDTA–glycerol solution (EDTA-G)

After fixation, the spinal column was washed for 12 h at 4 °C in each of the following series of solutions: 0.01 M PBS containing 5% glycerol, 0.01 M PBS containing 10% glycerol (Mallinckrodt Baker Chemicals: cat. # 5092), and 0.01 M PBS containing 15% glycerol (Miao and Scutt, 2002). The specimen was then decalcified in EDTA-G solution (14.5 g EDTA (Sigma–Aldrich cat. # ED2P), 1.25 g NaOH (ThermoFisher Scientific: cat. # S318), and 15% glycerol in 100 ml distilled pH 7.3. The EDTA-G solution was replaced every 5 days as previously described (Miao and Scutt, 2002). The progression of decalcification was checked at several time points using insertion of a sharp needle into the bone and chemical testing. After decalcification in EDTA-G, the specimen was washed at 4 °C for 12 h in successive washes of 15% sucrose and 15% glycerol in PBS, 20% sucrose and 10% glycerol in PBS, 20% sucrose and 5% glycerol in PBS, 20% sucrose in PBS, 10% sucrose in PBS, 5% sucrose in PBS, and 100% PBS.

2.2.4. 6% Trichloroacetic acid (TCA)

There are several published techniques using TCA to make a weak acid solution, mostly using a 5, 6 or 10% solution in distilled water. Previous studies have shown that 6% TCA decalcification preserves the immunoreactivity of laminin in bone (Graner et al., 1995a,b). Therefore, we assessed a 6% solution of TCA in distilled water. After fixation, the specimen was washed in PBS for 20 min. The whole vertebral column was then decalcified in 6% TCA (Sigma–Aldrich: cat. # T6399) in distilled water (this needs