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Autoclave treatment fails to completely inactivate DLB alpha-synuclein seeding activity

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ABSTRACT

Synucleinopathies are characterized by the deposition of alpha-synuclein (α-syn) aggregates in brain tissue. Pathological α-syn aggregates propagate in a prion-like manner and display prion-like biochemical properties. Using RT-QuIC, we measured α-syn seeding activity from brains of Dementia with Lewy body (DLB) patients post autoclave. Here, we show that autoclaving at 121 °C removes one to two $\log_{10}$ of α-syn seeding activity but the remaining 50% seeding dose (SD_{50}) is more than 107/mg tissue. DLB brain samples autoclaved at 132 °C still revealed an SD_{50} of approximately 106/mg tissue. Our data suggest that DLB α-syn seeds are incompletely inactivated by standard autoclave, thus highlighting the need for evaluating laboratory procedures that fully inactivate them.

1. Introduction

Synucleinopathies including Parkinson's disease (PD) and dementia with Lewy body (DLB) are characterized by the deposition of pathologically misfolded alpha-synuclein (α-syn) associated with neuronal or glial cells in brain tissue [1]. Pathological α-syn aggregates are known to propagate in a prion-like manner by acting as proteopathic seeds [2]. Prions are proteinaceous infectious agents that are composed of a pathological isoform of native prion protein, termed PrP^{Sc} [3]. Pathological α-syn aggregates and prions share biochemical properties such as detergent-insolubility and resistance to limited proteolysis [4]. Prions are notorious for their resistance to routine protocols that are used to effectively inactivate most other pathogens [5]. Misfolded α-syn aggregates are likewise resistant to inactivation methods such as formalin fixation and autoclave treatment [6,7].

Seeding activity for both α-syn and PrP^{Sc} aggregates can be detected by real-time quaking-induced conversion (RT-QuIC), which is an in vitro cyclic amplification technique initially developed to detect PrP^{Sc} with high sensitivity and specificity [8]. RT-QuIC has also been adapted for the detection of pathological α-syn aggregates from various synucleinopathies [9]. The RT-QuIC assay can be used for the quantitation of seeding activity in proteopathic seeds by determining those dilutions yielding positive responses in 50% of the replicates (SD_{50}) [8]. Relative amounts of seeding activity can be expressed as the SD_{50} concentration. In this study, we used α-syn RT-QuIC to quantitate α-syn seeding activity from DLB patient brain tissue after selected autoclave protocols. We additionally examined PrP^{Sc} seeding activity from the brain tissue of CWD-affected elk. Our results show that autoclaving at 121 °C for 20 min removes one to two $\log_{10}$ of α-syn seeding activity with the remaining 50% seeding dose (SD_{50}) more than 10^7/mg tissue, and that the removed seeding activity following this condition of autoclave are quantitatively similar between the two proteopathic seeds.

2. Materials and methods

Human brain tissue was obtained from the Edinburgh Brain and

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Table 1
Inactivation effects of autoclave treatment against α-syn or prion seeding activity.

Disease (protein)	Case ID in this study	EBTB's reference ID	Sex	Age at death (in years)	Brain region	α-syn concentration (μg/ml)	log_{10} SD_{50}/mg brain tissue (Mean ± SD) [1])		log_{10} SD_{50} decrease following steam sterilization (Mean ± SD)
							Untreated	121 °C	
DLB (α-syn)	DLB1	SD26/13	M	65	BA39 2,3)	1.1	9.70 ± 0.35	7.28 ± 0.12	2.42 ± 0.31
					BA9 4)	1.1	8.78 ± 0.24	7.12 ± 0.42	1.67 ± 0.42
	DLB2	SD19/14	M	60	BA9	1.6	8.82 ± 0.13	7.57 ± 0.38	1.25 ± 0.25
	DLB3	SD_{50}/12	M	61	BA9	2.1	8.95 ± 0.35	7.70 ± 0.20	1.25 ± 0.54
	DLB4	SD24/16	F	86	BA9	0.9	8.07 ± 0.13	7.20 ± 0.00	0.88 ± 0.13
CWD (prion)	C22-1	–	M	3	whole Brain	–	6.82 ± 0.13	5.45 ± 0.25	1.38 ± 0.18
	C22-2	–	F	5	whole Brain	–	6.32 ± 0.13	5.07 ± 0.38	1.25 ± 0.35
	C22-3	–	M	5	whole Brain	–	6.57 ± 0.13	5.20 ± 0.25	1.38 ± 0.18

1) Data shown represent the average values ± SD of two to three independent experiments.
2) In one of three experiments where a true end-point was not reached, we assumed that the next 10-fold dilution would have given 0 positive reactions in order to calculate the SD_{50} values.
3) BA39 = Brodmann area 39.
4) BA9 = Brodmann area 9.

Tissue Bank (EBTB) in Edinburgh, Scotland, UK. Brain samples derived from four patients with DLB (Brodmann area [BA] 9 and BA39) and from one non-demented control (frontal cortex [FC]) were used. Ethical approvals for the acquisition and use of human brain samples were obtained from the Institutional Review Board of Korea Brain Research Institute (KBRI). Elk brain tissue was obtained in a cervid farm in which CWD was identified in the Republic of Korea. Whole brains derived from three CWD-positive elk were used in this study. Positive CWD diagnosis for these animals was made by the Animal and Plant Quarantine Agency (APQA) in the Republic of Korea. Brain tissue was homogenized in PBS, pH 7.4, supplemented with complete EDTA-free protease and phosphatase inhibitors (Roche). Homogenization for CWD brains was achieved by manual grinding of a whole brain with mortar and pestle. DLB brain tissue was homogenized by using the Precellys 24 tissue homogenizer (Bertin instrument). The 10% (w/v) brain homogenates were clarified by centrifugation at 2000 g and then kept at −80 °C until needed. Brain homogenates were autoclave-treated either at 121 °C or 132 °C for 20 min and then stored at −80 °C until needed. Autoclaving of DLB homogenates was conducted using a laboratory autoclave ES-315 (TOMY) in KBRI, and autoclaving CWD homogenates was performed using HA-305 M (Hirayama) in APQA. Prior to autoclaving, the cap of tubes containing samples were slightly loosened to allow steam penetration.

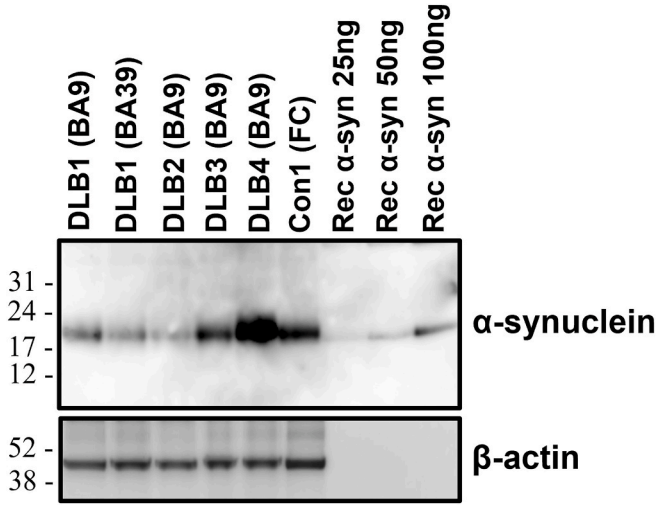

Fig. 1. Immunoblot analysis of α-syn in DLB brains. The brain homogenate from four DLB cases and one non-demented control case was subjected to immunoblot using the anti-alpha-synuclein antibody MJFR1, which recognizes residues 118–123 of α-syn. In each sample, 20 μg of total protein was loaded per lane. For quantitative estimation, recombinant α-syn protein at three different concentrations (25 ng, 50 ng, or 100 ng per lane) was included in the analysis. Molecular mass markers in kilodaltons were shown on the left.

The concentration of α-syn in the cleared 10% brain homogenates was measured using SensoLyte™ Anti-alpha-Synuclein Quantitative ELISA Kit (AS-55550-H, Anaspec). The homogenates were first diluted appropriately with sample dilution buffer supplied in the Kit to measure α-syn concentrations within the linear range of the standard curve, and then analyzed as described in the manufacturer's instructions.

For the Western blot analysis of α-syn, the cleared 10% brain homogenates were mixed with NuPAGE LDS sample buffer (Invitrogen) to a final concentration of 1X and incubated for 10 min in a heating block set at 100 °C. Proteins were separated in 12% Bis-Tris gels (Invitrogen) and then blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking with 0.2% I-Block (Applied Biosystems) in TBST (20 mM Tris-HCl[pH 7.4], 150 mM NaCl, and 0.1% Tween 20), the membranes were probed with anti-alpha-synuclein antibody MJFR1 (abcam) at a dilution of 1:10,000 or with anti-beta-actin antibody AC-15 (abcam) at a dilution of 1:5000. The membranes were then incubated with horseradish peroxidase (HRP)-labeled anti-rabbit IgG antibody (KPL 074–1516; for MJFR1) or anti-mouse IgG antibody (KPL 074–1806; for AC-15), both at a dilution of 1:10,000. The blots were developed either with Supersignal West Pico PLUS Chemiluminescent Substrate or TMB-Blotting Substrate Solution (Thermo Scientific).

RT-QuIC for the detection of α-syn seeding activity was conducted as described previously [9,10] with minor modifications. Briefly, 10% brain homogenates (10^{-1} dilution) were sonicated with 5 cycles of 30 s sonication and 30 s rest in Microsonix 4000 (Sonix) set at 80% potency, as described previously [11]. Sonication was conducted in Cup Horns in an indirect manner (i.e. without the use of probe). Subsequently, sonicated samples were subjected to serial tenfold dilutions in the homogenization buffer as indicated. Recombinant wild-type full-length human α-syn (rPeptide #S-1001-1) was used as substrate. Black 96-well plates with clear bottom (Thermo #265301) were preloaded with 4 glass beads (1.0–1.25 mm in diameter). 100 μL reaction mixtures containing 40 mM phosphate, pH 8.2, 10 μM ThT, 170 mM NaCl, 0.00125% SDS, 0.1 mg/mL recombinant human α-syn and 2 μL seeds at specified dilutions were loaded in quadruplicate, and then incubated at 37 °C in a FLUOstar Omega plate reader (BMG Labtech) with cycles of 1-min shake (400 rpm, double orbital) and 1-min rest for 50 h. ThT fluorescence in relative fluorescence units (rfu) was recorded at the starting point of the assay and then at 1-h intervals from bottom of wells (gain of 1,900, 440 nm excitation and 480 nm emission). Samples were deemed positive when two or more of four replicates exceeded the threshold fluorescence values, as determined by averaging fluorescence values of the first five measurements for all samples plus ten standard deviations. Protein

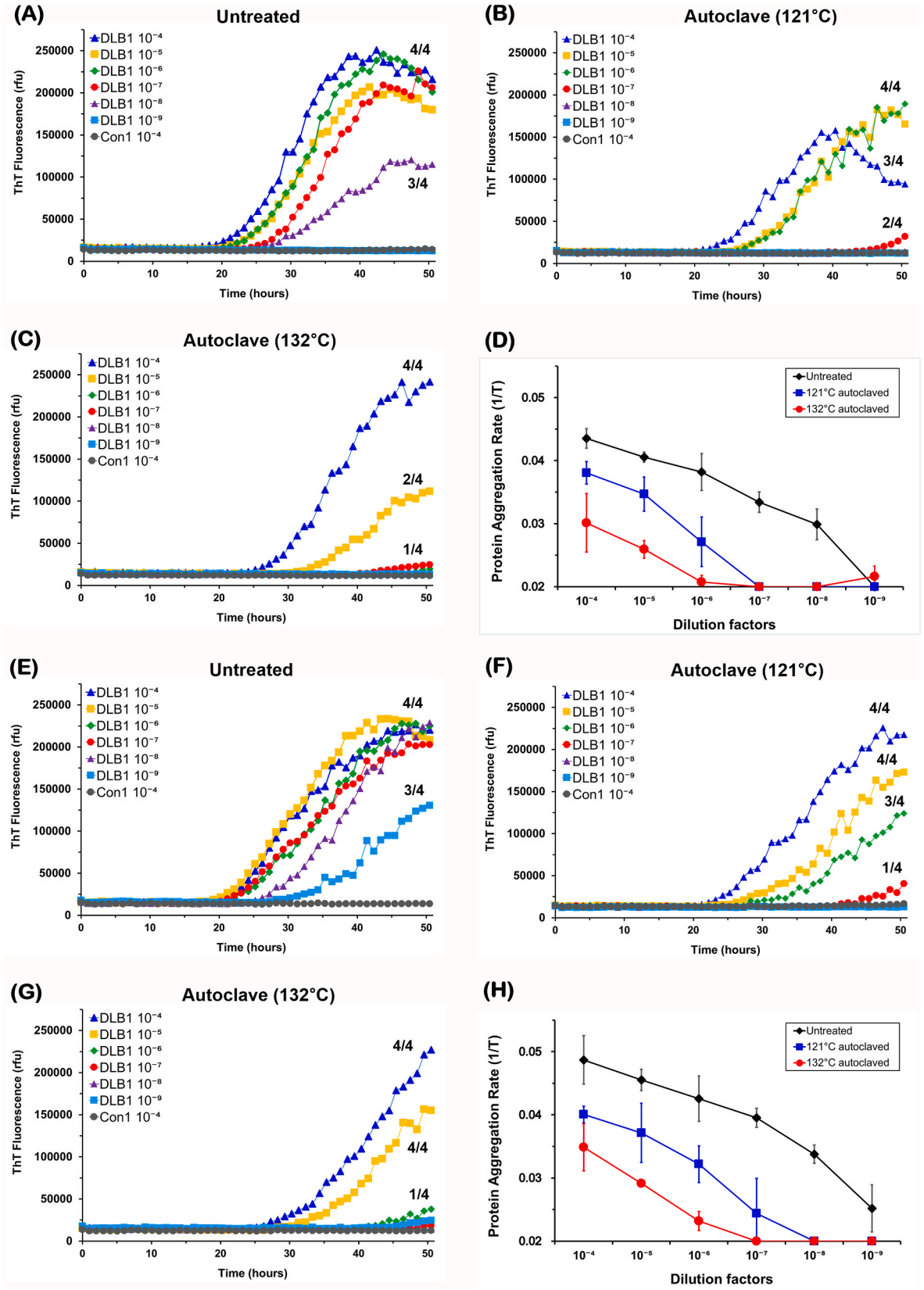

Fig. 2. RT-QuIC analysis of α-syn seeding activity in untreated or autoclaved DLB brain homogenates. DLB brain homogenates were prepared either from BA9 tissue (A, B, C, D) or BA39 tissue (E, F, G, H) of DLB1 patient. Frontal cortex homogenates from a non-demented patient (Con1) were used as a control. Brain homogenates remained untreated (A, E) or autoclaved for 20 min either at 121 °C (B, F) or 132 °C (C, G). Brain homogenates (2 μL) at indicated dilutions were used to seed α-syn RT-QuIC reactions in quadruplicate. Average ThT fluorescence values from quadruplicate wells were plotted against time (The negative replicates were taken for the average). (D, H) Protein aggregation rates (PAR) were obtained by taking the inverse of lag phase time (1/T). For reactions in which average ThT signals did not exceed the threshold values during the assay, lag phases were assigned as 50 h. Data shown represent the average values ± SD of three independent experiments.

aggregation rates (PAR) were obtained by taking the inverse of hours required to reach the threshold fluorescence (lag phase). SD_{50} values were calculated using the Spearman-Kärber method [8,10]. RT-QuIC for the detection of prion seeding activity was performed as described previously [12] with minor modifications. Briefly, 10% brain homogenates were serially diluted from 10^{-2} to 10^{-9}. A volume of 98 μL reaction mixtures (10 mM phosphate, pH 7.4, 1 mM EDTA, 10 μM ThT, 300 mM NaCl and 0.1 mg/ml recombinant Syrian hamster prion protein [residues 23–231, Impact Bio #201-01]) was loaded in quadruplicate, with 2 μL of diluted brain homogenates finally added to individual wells. The plate was incubated in the FLUOstar Omega plate reader at 42 °C for 71 h with cycles of 1-min shake (700 rpm, double orbital) and 1-min rest. Samples were deemed positive when at least three of four replicates exceeded 20,000 rfu.

3. Results and discussion

We first measured α-syn concentrations by ELISA in the brain homogenates used for seeding. Concentrations of α-syn were measured in the range of 0.9 μg/ml to 2.1 μg/ml in the 10% homogenates, showing some variation between samples (Table 1). Similarly, immunoblot band intensities of α-syn migrating approximately at 18–19 KDa also displayed minor variation between samples (Fig. 1). We then examined α-syn seeding activity in non-autoclaved DLB brain homogenates with serial dilutions up to 10^{-11}. Flat responses were seen in control (Con1) reactions and positive responses rising above baseline were detected in reactions with 10^{-4} to 10^{-8} DLB1 BA9 tissue dilutions (Fig. 2A) or 10^{-4} to 10^{-9} DLB1 BA39 tissue dilutions (Fig. 2E). The SD_{50} values reached 8.78 $\log_{10}$ SD_{50}/mg tissue (BA9) or 9.7 $\log_{10}$ SD_{50}/mg tissue (BA39), respectively. We then analyzed α-syn seeding activity in the BA9 tissue of three more DLB cases. Positive RT-QuIC responses were detectable in reactions seeded with up to 10^{-7} to 10^{-8} dilutions, with relative concentrations of α-syn seeding activity in the range of 8–9 $\log_{10}$ SD_{50}/mg tissue (Table 1).

Next, brain homogenates from DLB1 patient were autoclaved either at 121 °C or 132 °C for 20 min prior to RT-QuIC. At 121 °C, positive responses were identified in reactions seeded with up to 10^{-7} dilution of BA9 tissue but were not detectable with tissue dilutions of 10^{-8} to 10^{-9} (Fig. 2B). The remaining α-syn seeding activity was $10^{7.12}$/mg tissue. At 132 °C, α-syn seeding activity was identified only in reactions seeded with 10^{-4} to 10^{-5} dilutions of BA9 tissue with the SD_{50} value of $10^{5.95}$/mg tissue (Fig. 2C). When compared to non-autoclaved BA9 homogenate, SD_{50} values were reduced by 1.67 $\log_{10}$ following autoclave at 121 °C or by 2.83 $\log_{10}$ following 132 °C. Protein aggregation rates (PARs) were calculated as the inverse of the lag phase. Temperature-dependent reductions in PAR were observed at each dilution following autoclave treatment (Fig. 2D). Similar RT-QuIC kinetics were observed for the DLB1 BA39 homogenates (Fig. 2F, G and H). Following autoclave of the DLB1 BA39 homogenates, seeding activity titers decreased by 2.42 $\log_{10}$ (at 121 °C) or 3.13 $\log_{10}$ (at 132 °C) when compared to the corresponding non-autoclaved sample. The analysis of BA9 tissue from three additional DLB cases autoclaved at 121 °C showed similar results to DLB1, with the reduction of SD_{50} values in the range of 0.88–1.25 $\log_{10}$ (Table 1).

We then investigated prion seeding activity in CWD-positive elk brains before and after autoclave treatment. The SD_{50} values in non-autoclaved brain homogenates from CWD-positive elk were in the range of 6–7 $\log_{10}$ SD_{50}/mg tissue (Table 1). Following autoclave treatment at 121 °C for 20 min, SD_{50} values were reduced by 1.25–1.38 $\log_{10}$ when compared to the corresponding non-autoclaved samples and the remaining prion seeding titers reached 5 to 5.5 $\log_{10}$ SD_{50}/mg tissue.

In this study, we quantitated the decrease of seeding activity in autoclaved DLB α-syn aggregates or CWD prions using end-point RT-QuIC [8]. Our results show that autoclaving at 121 °C removes one to two $\log_{10}$ of α-syn seeding activity but the remaining seeding activity titer is more than 10^{7}/mg tissue. Consistent with our data, α-syn seeding activity from other synucleinopathies such as PD or multiple system atrophy (MSA) has also been reported as highly resistant to physiochemical inactivation. Tarutani and colleagues showed that MSA α-syn seeding activity measured using a cellular system was shown to be reduced by approximately 1 $\log_{10}$ SD_{50} following autoclaving at 134 °C for 20 min [6]. Another study performed by Pinder and colleagues reported that autoclaving PD brain homogenates at 134 °C removed 2.2 to 2.4 $\log_{10}$ of α-syn seeding titers irrespective of autoclave time [13]. Thus, our data suggest that α-syn seeding activity in DLB brains is more susceptible to inactivation by autoclave than that of PD or MSA brains. Notably, pathogenic α-syn aggregates derived from DLB, PD or MSA brains have been reported to differ in their physiochemical and biological properties [14–17]. While further investigations are needed to more precisely quantify the levels of resistance of α-syn seeds from various synucleinopathies, remaining α-syn seeding activity after standard autoclave might be a public health problem given the structural, biochemical and mechanistic similarities between PrP^{Sc} and other proteopathic seeds [18,19]. There are growing concerns that neurodegenerative disorders such as Parkinson's and Alzheimer's disease (AD) might be transmissible between humans [20]. In fact, multiple cases of iatrogenic amyloid β transmission have been reported in recent years [21,22]. To the best of our knowledge, there is so far no evidence of α-syn transmission between humans. Nonetheless, incomplete inactivation of α-syn aggregates after standard autoclave procedures could be a concern in hospital environments. Therefore, more effective protocols for pathological α-syn aggregate inactivation are needed to fully remove any risk associated with human-to-human transmission. These inactivation methods could include treatments with 1–2 N sodium hydroxide, 20,000 ppm sodium hypochlorite, vaporized hydrogen peroxide, a mildly acidic formulation of hypochlorous acid or acidic SDS, and prolonged autoclaving at higher temperature [5,23–27], all of which were found to be highly effective in decontaminating prions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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