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Evaluation of cold atmospheric plasma for the decontamination of flexible endoscopes

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SUMMARY

Background: Despite adherence to standard protocols, residues including live micro-organisms may remain on the various surfaces of reprocessed flexible endoscopes. Prions are infectious proteins that are notoriously difficult to eliminate.
Aim: To test the potential of cold atmospheric plasma (CAP) for the decontamination of various surfaces of flexible endoscopes, measuring total proteins and prion residual infectivity as indicators of efficacy.
Methods: New PTFE endoscope channels and metal test surfaces spiked with test soil or prion-infected tissues were treated using different CAP-generating prototypes. Surfaces were examined for the presence of residues using very sensitive fluorescence epimicroscopy. Prion residual infectivity was determined using the wire implant animal model and a more sensitive cell infectivity assay.
Findings: A CAP jet applied perpendicularly at close range on flat test surfaces removed soil within 3 min, but left microscopic residues and failed to eliminate prion infectivity according to the wire implant animal assay. The longitudinal gas flow from CAP prototypes developed for the treatment of long channels led to the displacement and sedimentation of residual soil towards the distal end, when applied alone. Observations of the plasma inside glass tubes showed temporal and spatial heterogeneity within a limited range. After the standard enzymatic manual pre-wash, 'CAP-activated' gas effluents prevented prion transmission from treated endoscope channels according to the prion infectivity cell assay.
Conclusion: CAP shows promising results as a final step for decontamination of surgical surfaces. Optimizing CAP delivery could further enhance CAP efficacy, offering a safe, chemical-free alternative for the reprocessing of all luminal flexible endoscope surfaces.

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Introduction

Modern flexible endoscopes are relatively expensive and complex reusable instruments that are commonly used for diagnostic and surgical interventions, some of which contradict their classification as 'semi-critical devices' [1]. Different accessories can be inserted through the working channel, causing abrasions and creating grooves which facilitate soil adsorption [2]. Consequently, these channels retain residues from patient fluids, microbial flora and other tissues depending on the surgical site and type of interventions involved. In addition, protein deposits may facilitate the adsorption of bacteria, and any delay in reprocessing allows partial dehydration and further enhances the retention of these complex soils [3].

Luminal flexible endoscopes cannot be autoclaved. Their common reprocessing regimen consists of a bedside rinse immediately after use, prior to transfer to a specialized facility. There, manual washing relying on longitudinal brushing and/or flushing of the various channels is followed by automated washing and high-level disinfection in an automated endoscope reprocessor. There is evidence that these processes do not remove all contamination, even when performed in accordance with the manufacturers' instructions [4—6]. Microbial recolonization after reprocessing is a well-known problem. Reprocessing endoscopes before use following 'extended storage' is common practice, although local procedures and national recommendations vary [7]. Existing tests to assess the cleanliness of endoscopes rely on flushing the channels to detect biological indicators, such as ATP or culturable bacteria, in the eluate. Despite the questionable sensitivity of such tests [8], positive results which may only show the 'tip of the iceberg' are accepted up to a certain threshold, which again varies between locations [7]. As a result of these limitations, cross-contamination between successive patients may occur. The increasing demand for preventive and curative endoscopic interventions coincides with the development of antimicrobial resistance worldwide, and recent outbreaks involving antimicrobial-resistant pathogens have brought this issue to light [9—16]. In recent years, ethylene oxide has become an additional treatment of choice, particularly in the USA, despite concerns for clinical staff, time and financial cost [17,18]. This raises questions about the future benefit vs risk balance of some endoscopic interventions while adhering to current procedures.

The identification of prions as the infectious agent of transmissible spongiform encephalopathies (TSEs) [19] led to a number of new concerns regarding the decontamination of reusable surgical instruments, particularly in the UK which was affected by variant Creutzfeldt—Jakob disease (vCJD, a TSE affecting humans) in the mid-1990s [20]. Pathogenic prions (PrP^{Res}) are amyloid-rich, hydrophobic, protease-resistant proteins which aggregate in the central nervous system, leading to invariably fatal neurodegeneration. While no vCJD transmission through endoscopy has been reported to date, the tissue distribution of infectivity in asymptomatic vCJD carriers implies a risk of transmission whenever nerves within lymphoreticular tissues are accessed. Due to the increasing complexity of instruments and difficulty associated with cleaning some parts, including long lumens, the removal of potential prion contamination and/or elimination of prion infectivity from these instruments could be considered the most difficult target in the field of decontamination [21]. Given the perceived limitations of current reprocessing technologies and the absence of an established non-invasive preclinical test for vCJD, the UK and other countries still follow precautionary measures, and endoscopes used on identified vCJD carriers are quarantined or destroyed at some significant cost [22—24].

Low-temperature sterilization methods such as vaporized gases appear promising to address potential prion contamination in endoscopes [25]. Developments in cold atmospheric plasma (CAP) technologies allow the treatment of surfaces at ambient pressures without generating excessive heat, making CAP another potential tool for the decontamination of endoscopes [26]. Gas plasma is a transient state of matter harbouring various short-lived ionized gas species which have shown promising antimicrobial capabilities against biofilm models grown inside plastic channels and other clinically relevant surfaces [27,28]. Furthermore, the short lifespan of these reactive species precludes the need for large volumes of rinsing water, and reduces the risks of exposure to patients, clinical staff and the environment, making CAP an attractive alternative to chemical treatments.

This study evaluated CAP for the decontamination of luminal endoscope surfaces, particularly the lumen of working channels up to 1200 mm, and the potential effect of CAP on residual prion infectivity inside those channels.

Methods

Generation of cold atmospheric plasma

The first CAP prototype tested generated a plasma plume (up to 2 cm; Figure 1A) from a helium/oxygen gas mixture using pulse discharge (1.3 kV at a modulation frequency of 25 kHz), as described elsewhere [29]. Contaminated 316L grade stainless steel tokens and stainless steel or polyurethane-insulated silver wires were treated in a biosafety cabinet within range of the plasma plume, where the concentration of ionized species is expected to be highest.

The second CAP prototype tested (co-axial CAP) was sealed to the proximal end of test channels to inject the plasma inside (Figure 2A) [28]. Another tube was sealed at the distal end to collect effluents into a bleach container. In this system, plasma was produced inside a quartz tube by applying voltage of 10 kV and electrical power of 15.9 W at 23 kHz to a 4 standard litres per minute (SLM) flow of argon (99.9%), which was found previously to be the optimal condition for plasma generation.

Contamination and treatment of metal surfaces

Polished 316L stainless steel flat tokens were cleaned thoroughly in acetone and autoclaved. Tissue homogenates (control and 22L-infected C57BL/6J mouse brain homogenate; TSE Resource Centre, The Roslin Institute, University of Edinburgh, UK) were assayed for protein content (Bradford assay) and normalized to 1 mg/mL total protein in phosphate-buffered saline (PBS, Sigma, St Louis, MO, USA) [30]. One microlitre was applied to cleaned tokens and dried for 1 h at room temperature prior to CAP treatment or direct staining for controls. Tokens were kept on a plastic surface during treatment.

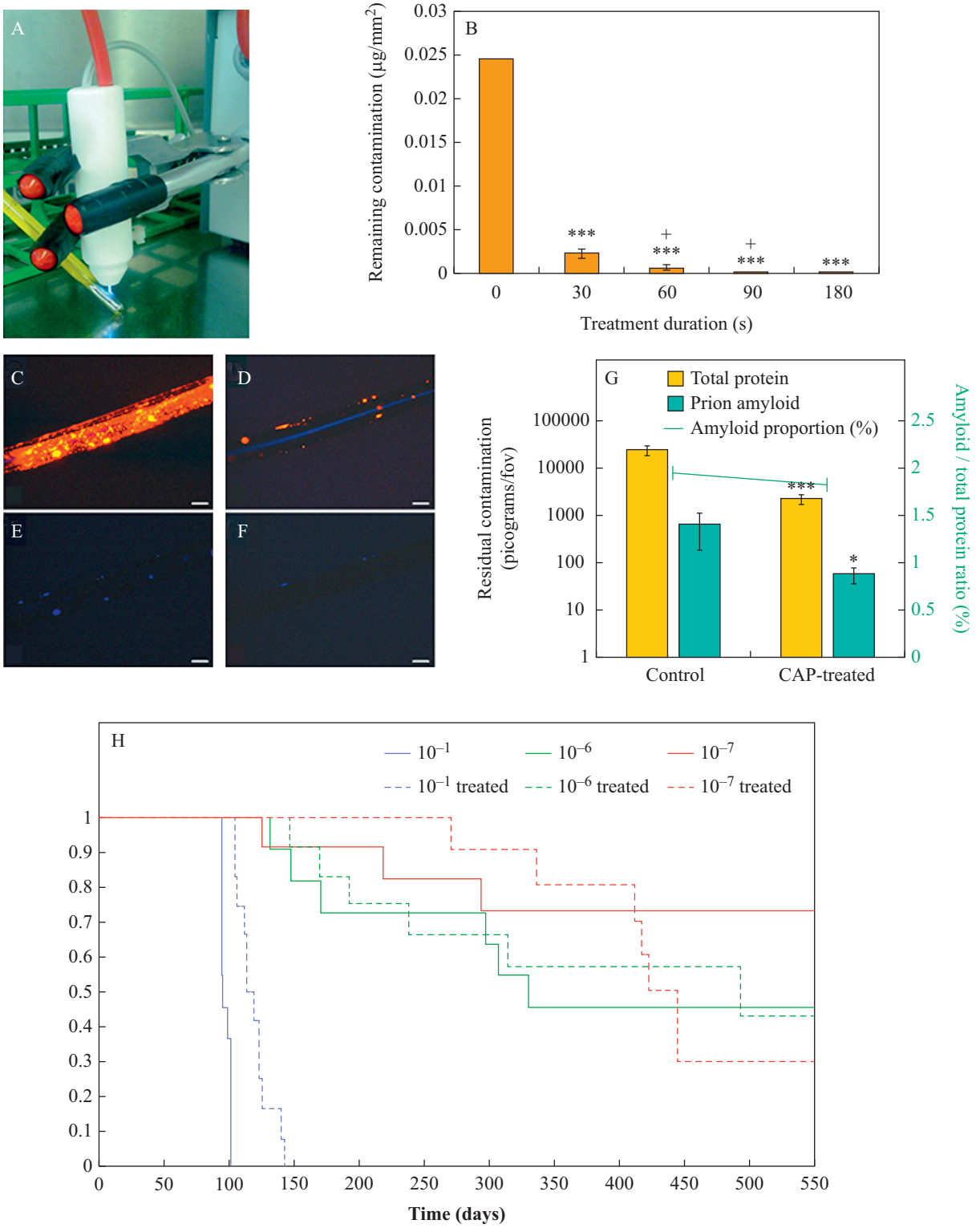


Figure 1. (A) The first cold atmospheric plasma (CAP) prototype tested which produced a small plasma plume. Maximum temperature measured directly as shown in the picture was 44 °C. (B) Reduction profile of contamination observed on stainless steel tokens spiked with the same amount of brain homogenate and exposed to CAP plume for various durations [mean ± standard error of the mean (SEM); *N*=3]. (C–F) Episcopic differential interference contrast microscopy combined with epifluorescence pictures of wires contaminated with 10% 263K-infected hamster brain homogenate, showing total protein stained with SYPRO Ruby before treatment (C) and after CAP (D), and thioflavin T staining of amyloid before (E) and after (F) CAP treatment. Bars are 100 µm. The blue line visible in (D) is excitation light reflected from the wire surface. (G) Mean ± SEM of detected contamination on control (*N*=11) and CAP-treated wires (*N*=16). (H) Survival distribution curve from the wire implant assay.

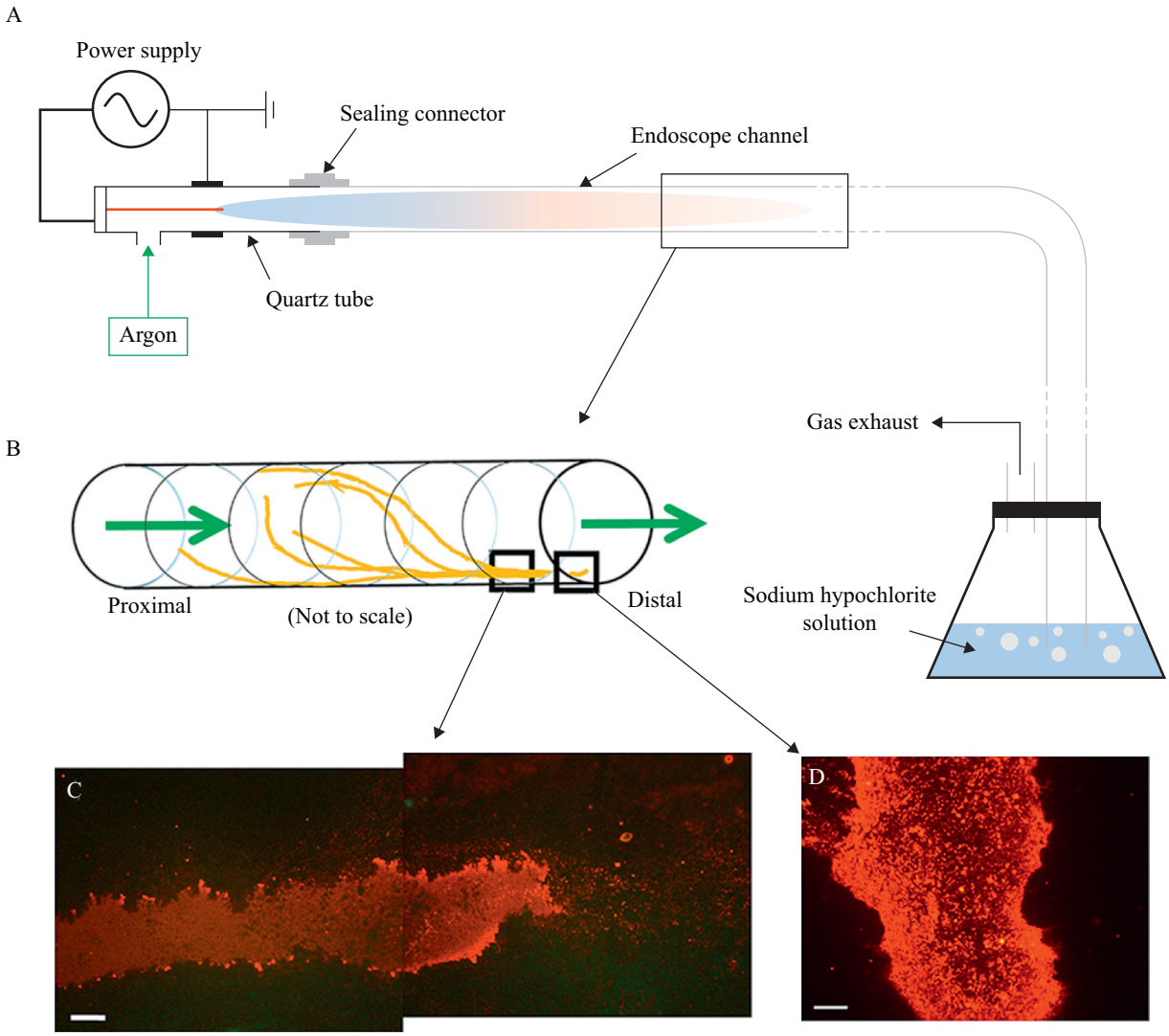


Figure 2. (A) Diagram of the plasma set-up tested. Argon is injected inside a quartz tube with one central and one annular electrode. High voltage (10 kV) generates cold atmospheric plasma (CAP) which is forced inside the sealed channel of an endoscope. (B) Diagram summarizing protein residues observed within channels after CAP treatment without pre-wash. Soil was displaced (amber line paths) by the relative high velocity of gas flow (green arrows) forming a smeared line, possibly with the contribution of gravity (C). (D) Transverse deposit observed at the distal end. Bars are 100 μm.

Contamination and treatment of wires for animal infectivity assays

For the in-vivo infectivity assay, polyurethane-insulated silver wires (0.16 mm diameter, 5 mm length; GoodFellow, Huntingdon, UK) were cleaned thoroughly in acetone and autoclaved. Wires were then immersed for 1 h into various 10-time serial dilutions of a 10% (w/v in sterile PBS) 263K-infected brain homogenate into healthy, normal brain homogenate (all tissues from TSE Resource Centre). The highest prion concentration in tissues consisted of the original 10% brain homogenate in PBS (labelled 10^{-1} dilution); the 10^{-6} dilution (for which a longer mean incubation period was expected) and the 10^{-7} dilution (the detection limit of this assay according to previous studies) were also tested. Wires were then dried for 16 h before rinsing three times in sterile PBS for 5 min to remove non-specific materials bound to the surface. For each dose, two wires were contaminated (for CAP jet treatment or respective control). Wires were held under the plasma plume and moved along to achieve 2 min of treatment on the whole surface (assessed visually), which was the minimum exposure time providing consistent good removal from flat surfaces. Each wire was then cut into 10 sections of the appropriate size (tips excluded for handling purpose). Wire sections were anonymized, packaged individually under sterile conditions, and sent for cranial implantation into susceptible animals as described by Fichet *et al.* [31] to the Commissariat à l'Énergie Atomique which had appropriate ethical approval for this study (Reference Nos A92-032-02 and 92-189). Clinical observation was undertaken until the animals died or were culled 550 days post implantation. LD_{50} values were determined according to the Reed and Muench method [32]. Diagnosis of TSE was confirmed by detection of PrP^{Res} in brain by enzyme-linked immunosorbent assay and Western blot techniques.

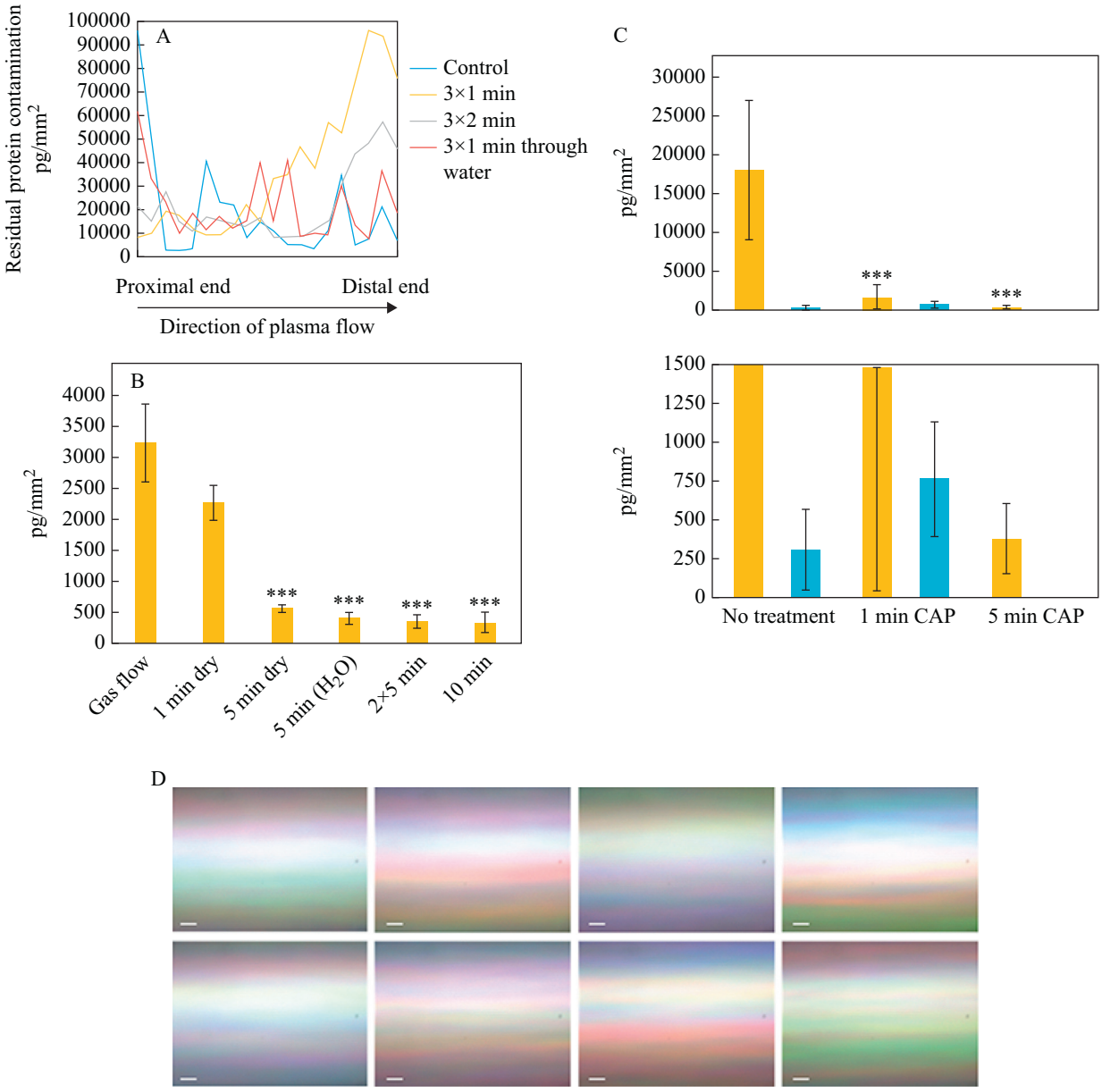


Figure 3. (A) Profiles of protein deposition in endoscope channels after cold atmospheric plasma (CAP) treatment of various durations. (B) Total proteinaceous residues in endoscope channels after CAP treatment of various durations [mean ± standard error of the mean (SEM) from 12 separate experiments]. (C) Profile of amyloid (blue bars) and total protein (orange bars) removal by 1 and 5 min of CAP treatment after pre-rinse ('no treatment') using experimental tubing provided by Old Dominion University spiked with Scrapie sheep blood (mean ± SEM from three separate experiments). (D) CAP (4 standard litres per minute) glow discharge at a fixed location (approximately 10 cm from the electrodes where CAP is generated) inside transparent endoscope working channels as seen under episcopic differential interference contrast microscopy. Bars are 100 μm.

Contamination and treatment of endoscope channels

New endoscope channels (internal diameter 3.2 mm) purchased from a global manufacturer were contaminated by injecting Browne test soil (Medisafe, Bishops Stortford, UK) or murine 22L Scrapie-infected brain homogenates produced in a parallel project (Project License No. 30/3056; Matthew Davies Home Office License No. 30/9788, with appropriate ethical approval) [33], using a sterile syringe sealed at the proximal end of the channel. Excess contamination was washed off by flushing sterile water once using a new syringe. Without drying, contamination adsorbed to the luminal surface of the channels was subjected to one of three different conditions: (i) an Enzol (Johnson and Johnson, Wokingham, UK) wash alone according to the manufacturer's instructions; (ii) a 2-min CAP treatment alone; and (iii) an Enzol wash followed by a 2-min CAP treatment. Effluents from the treated channels were recovered through a solution containing 20,000 ppm sodium hypochlorite (Figure 2A).

Staining and microscopy

Channels and wires were dual stained for prion amyloid and total proteinaceous contamination using Thioflavin T (ThT; Sigma) and SYPRO Ruby (SR; Invitrogen, Waltham, MA, USA),

respectively, as described elsewhere [30]. Channel sections were cut in half longitudinally, and excess water was dried off prior to observation under episcopic differential interference contrast microscopy combined with epi-fluorescence (EDIC/EF; Best Scientific, Swindon, UK). ThT and SR signals were quantified from at least 10 representative fields of view for each sample examined using Image Pro (MediaCybernetics, Silver Spring, MD, USA).

Cell-based infectivity assay

A dynamic cell-based infectivity model developed in the authors' laboratory, based on the Scrapie cell end point assay, was used in this study [34,35]. Briefly, appropriate prion-susceptible cells were grown in the presence of prion-contaminated surfaces. The N2a#58 cell line (a kind gift from S. Lehmann, Montpellier, France) was used to detect residual 22L prion infectivity in transverse sections (1-cm length) of the distal end of treated or control endoscope channels cut longitudinally into quarters. Near confluency, the contaminated surfaces were transferred with adherent cells, grown again to confluence. The surfaces were removed, and cells were maintained through successive passages in several dishes. At each passage, some cells were fixed and stained using Sudan Black and ThT to quantify prion amyloid accumulation in cells using EDIC/EF microscopy [34].

Observation of cold atmospheric plasma flow in channels

To examine the flow of plasma inside a channel, a 10-cm piece of glass tube of fitting diameter was sealed between two pieces of flexible endoscope channel, with the proximal end connected to the plasma generator (within range of the visible plasma discharge) and the distal end connected to the outlet. The glass tube was held in place under an episcopic microscope using tape. The plasma glow discharge at the same location was filmed, and instant images were captured used bright light EDIC microscopy.

Statistics

The cleaning efficacy of CAP against amyloid specifically and all proteins was compared with corresponding controls using Student's *t*-test (SPSS; IBM Corp., Armonk, NY, USA). $P \leq 0.05$ was considered to indicate significance.

Results

Action of short-range cold atmospheric plasma against proteinaceous contamination and prion infectivity on wires

The short-range CAP design was very effective at removing proteins from flat surfaces exposed perpendicularly to the plasma plume, achieving a 3 log reduction in residual proteins from a 1-μg dried deposit after 90 s of treatment (Figure 1B). The CAP plume appeared less efficient at removing residues from the convex, rod-shaped stainless steel wires, although ThT/SR dual staining of incompletely decontaminated wires suggested that CAP treatment removes both amyloid and other proteins with the same efficacy (Figure 1C—G). Similarly, CAP treatment, applied as described above, failed to eliminate prion infectivity bound to polyurethane-insulated silver wires used in the animal assay. The survival distribution (Kaplan—Meier) curves obtained from the wire implant test (Figure 1H) shows that CAP treatment only delayed the transmission of infectivity moderately from wires originally contaminated with a relatively high titre of 263K prion-infected brain (10^{-1} dilution of original brain tissue). Using a 5-log dilution (10^{-6}), residual infectivity appeared to remain similar before and after treatment. For the lowest concentration of prion in brain tissues (10^{-7}), after a delay in the appearance of the first positive cases (which motivated extending the observation period to 18 months), the transmission rate was increased following CAP treatment.

Action of long-range co-axial CAP against proteinaceous contamination within endoscope channels

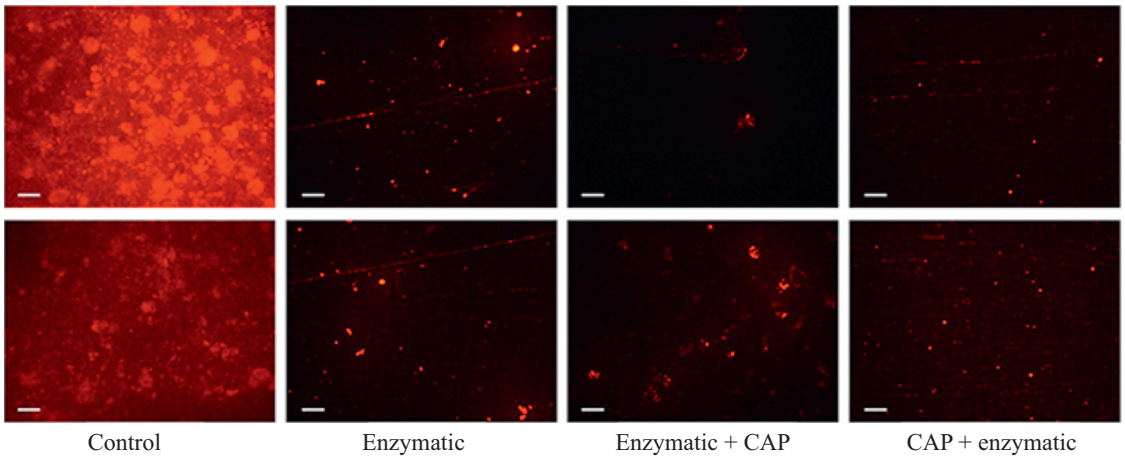
In this configuration, the plasma moves tangentially to the contaminated surface. Using this system, the plasma discharge observed inside channels was approximately 20 cm.

After a single rinse to remove excess Browne soil, co-axial CAP treatment of endoscope channels led to the smearing of soil residues on the luminal surface (Figure 2B and 2C) and areas of transversal deposition towards the distal end of the channels (Figure 2D). Other CAP prototypes which also relied on injecting gas within the channel prior to CAP ignition through various electrode arrangements were tested, and smearing of proteinaceous residues was observed in all cases (data not shown). Observations of the smears in different sections of the channel suggest that this was caused by the combined effect of gas flow and gravity (Figure 2B). While the gas flow was maintained, extending the duration of CAP treatment (three repeats of 1 or 2 min) only partly reduced the amount of residual soil found in such aggregates. Attempts to pass the gas through water to increase relative humidity prior to plasma ignition produced additional aggregates closer to the proximal end (Figure 3A). No further reductions in total residual proteins within the channels were measured beyond 5 min of CAP treatment. Increasing the relative humidity had no significant effect on the overall process (Figure 3B). This apparent flocculation of soil within contaminated channels (without preliminary washing) may be specific to plasma or other gas treatments, whereas water-based reprocessing should facilitate dissolution and flushing of most contamination in heavily soiled channels.

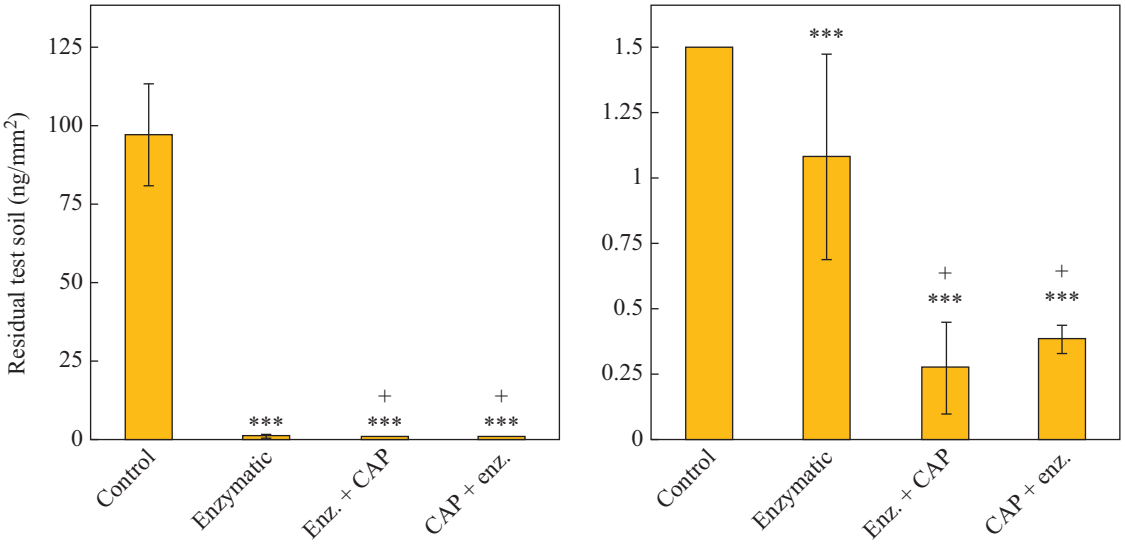
When measuring mean residual proteins from various sections from the total length of treated channels (excluding aggregates), CAP treatment alone resulted in a 2-log reduction in total residual proteins. Performing the same tests on channels spiked with Scrapie-infected blood, more amyloid proteins could be detected after 1 min of treatment, which was in accordance with previous observations using the dual staining protocol, whereby the specific fluorescence from ThT bound to amyloid aggregates may be partly masked by other soil constituents [30]. However, after 5 min of CAP treatment, the mean residual protein load within channels was further reduced and no amyloid aggregates were detectable by ThT staining in these experiments (Figure 3C).

Observation of the plasma glow discharge and sequential microphotographs showed spatial and temporal variations in

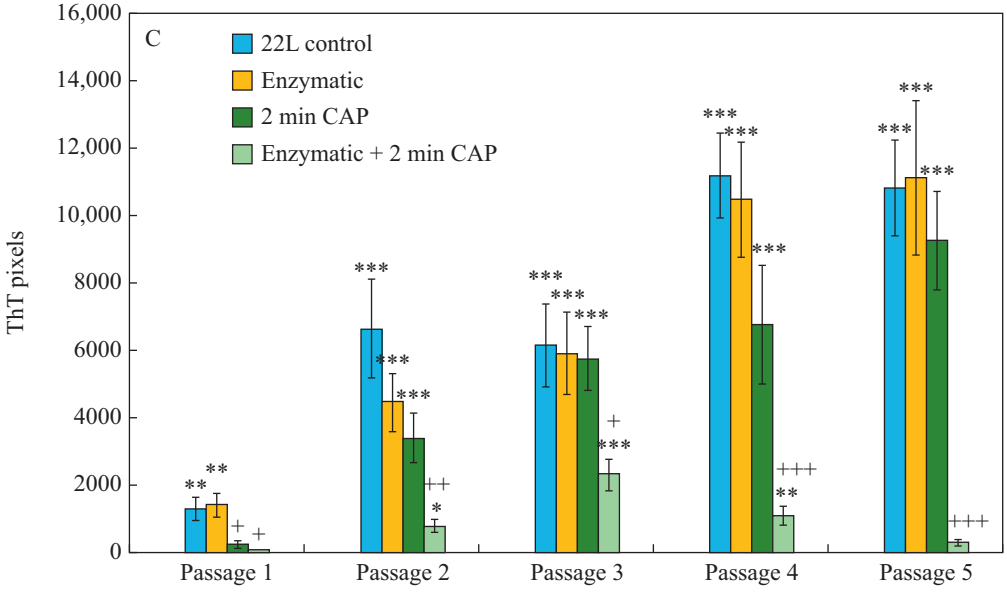
A


Control
Enzymatic
Enzymatic + CAP
CAP + enzymatic

B


Residual test soil (ng/mm²)
125
100
75
50
25
0
1.5
1.25
1
0.75
0.5
0.25
0
***
+
Control
Enzymatic
Enz. + CAP
CAP + enz.

C


C
22L control
Enzymatic
2 min CAP
Enzymatic + 2 min CAP
ThT pixels
16,000
14,000
12,000
10,000
8000
6000
4000
2000
0
Passage 1
Passage 2
Passage 3
Passage 4
Passage 5

the distribution of ionic species, based on rapid variations in the visible light emission wavelengths (Figure 3D).

Combined action of co-axial cold atmospheric plasma and enzymatic cleaning against proteinaceous contamination and prion infectivity within endoscope channels

Combining enzymatic cleaning and CAP treatment against Browne soil produced a synergistic effect for total protein removal from endoscope channels (Figure 4A,B). Since applying CAP after enzymatic cleaning appeared to produce the best results, this combination was tested against prion infectivity.

This cell-based infectivity assay confirmed previous findings that this enzymatic treatment alone, although very efficient at removing proteins, does not eliminate residual prion infectivity. Two minutes of CAP treatment alone was also inefficient at eliminating prion infectivity, as proteins remained. Using CAP as an add-on treatment following enzymatic treatment prevented stable and sustained prion propagation within N2a#58 following cell passages, and amyloid accumulation was reduced beyond passage 4 (Figure 4C).

Discussion

The action of CAP against soil adsorbed on surfaces includes two main aspects. The gas flow itself may displace gross contamination, and concomitantly, the ionized short-lived reactive species can damage bacteria and virus membranes and potentially alter molecules such as prions, rendering them innocuous.

This study found that CAP alone was moderately efficient at removing adsorbed soil, particularly when applied tangentially to the target surfaces, which is pertinent to the decontamination of endoscope channels. The geometry of wires may also affect the mechanical action of the gas flow or any similar treatments. In preliminary experiments, better cleaning results, in terms of protein removal, were observed when using CAP on flat surfaces. These observations of treated stainless steel flat surfaces suggest that a CAP plume can remove amyloid and other proteins with the same efficacy. This is an improvement compared with the action of many water-based chemistries, which were found to be less efficient against the amyloid fraction of prion-infected brain tissues, resulting effectively in partial purification of amyloid aggregates adsorbed on stainless steel surfaces [21]. However, microscopic observations showed that minute residues remained on CAP-treated wires despite efforts to apply CAP homogenously. The animal infectivity assay confirmed the presence of residual prion infectivity on polyurethane-insulated silver wires treated in the same way. Similar results were reported in a study using hot plasma, where the authors proposed that dehydration of the prion aggregates resulted in reduced bioavailability of infectious material early after treatment, which led to delayed, increased transmission of infectivity [36]. Unlike hot plasma, CAP avoids excessive heating of the surfaces. While the flow of different gas mixtures with or without plasma may contribute to dehydrating adsorbed soil, the samples used in the present study were not implanted before several days, so dehydration is a possible explanation. Etching is another mechanism which may explain the infectivity results from treated wires. Partial degradation of amyloid aggregates through etching could result in the production of smaller amyloid prion aggregates that are undetectable under EDIC/EF (with a threshold of approximately 100 fg of protein). Smaller prion aggregates (down to fibril size) have been reported to be potentially more infectious, a feature used in in-vitro assays such as protein misfolding cyclic amplification, although the time course and pathophysiology of prion infection in the central nervous system is still unclear. It is also not clear whether smaller prion aggregates have similar functional properties to plasma-reduced aggregates of similar size. One limitation of the study protocol is that it was not possible to stain and inspect each polyurethane-insulated silver wire after CAP treatment and prior to the animal assay as the presence of amyloid-bound ThT may have interfered with the assay. As such, CAP efficacy on these wires was assumed to be identical to that observed in preliminary tests on similar and stainless steel wires.

Gas injected at the required flow rate (4 SLM) for CAP treatment within soiled endoscope channels was found to displace residual soil towards the distal end of the channel. The impact of proteinaceous deposits on micro-organism retention is unclear. Soil adsorbed inside endoscope channels may produce favourable grounds for the early development of biofilms. Protein deposits may also embed bacteria and protect them from chemicals or CAP itself. Furthermore, CAP activity is through the generation of short-lived reactive species, which must reach the target in sufficient concentration to effectively kill micro-organisms and/or inactivate pathogenic molecules such as prions. Residues accumulated over several months of repetitive use in old endoscope channels are more difficult to remove using existing reprocessing regimens [2]. Experimental and clinical evidence also shows that delivering effective concentrations of antimicrobial compounds is more difficult when targeting established biofilms with complex three-dimensional structures. Similar limitations may apply to CAP when treating soiled endoscope channels.

Despite the relatively high flow used with the co-axial system, the range of CAP itself (judging by visible discharge) was seen to be limited to approximately 20 cm within endoscope channels with a standard internal diameter (3–4 mm). Research groups in the field are increasingly focusing on the concept of 'plasma-activated gas' (PAG) or 'plasma-activated water' (PAW), which may facilitate the application of CAP in the decontamination field. Bhatt *et al.* reported promising results against simple bacterial contamination located at the distal end of model channels using the same co-axial CAP design, and attributed this effect to PAG [28]. Several questions remain about the safe use and limitations of CAP, PAG or PAW for the reprocessing of complex soils on various materials

Figure 4. Example micrographs (A) and residual protein profiles (B) [mean ± standard error of the mean (SEM) from three separate experiments] following individual or combined enzymatic wash and cold atmospheric plasma (CAP) treatment against Browne test soil in endoscope working channels. Bars on micrographs are 100 μm. The graphs show two scales for easier comparison. (C) Action of individual or combined enzymatic wash and CAP treatment against 22L prion infectivity in endoscope channels measured using the N2a#58/22L cell assay. Data show mean ± SEM from three independent experiments. ThT, thioflavin T.

found in surgical instruments, as potential interactions with some materials or residues could have adverse effects [37]. In the study system, CAP (or PAG) alone did not eliminate residual prion infectivity from tissue deposits at the distal end of endoscope channels. As discussed above, this indicates that reactive species generated by CAP may not reach or penetrate through soil aggregates, suggesting that CAP should be used as an add-on treatment on pre-washed channels. The enzymatic wash, although also insufficient on its own to eliminate all prion infectivity, contributed to removal of a significant amount of soil, facilitating CAP action according to the infectivity data obtained using the cell assay after combining both treatments.

In conclusion, flexible luminal endoscopes remain complex, delicate and costly instruments, and it is unlikely that manufacturers will ever produce viable single-use alternatives. Improving the cleaning and removal of biologically active contaminants from those instruments appears to be the main area of intervention to make endoscopy safer in the future. Interest and research into potentially longer lasting 'plasma-activated' fluids is developing in the field of decontamination. While the mode of action of CAP and its impact on various prion strains and complex soil residues such as bacteria/protein mix in older channels remain to be examined, the characteristics of CAP make it a potential tool for the final sterilization stage of such delicate surfaces, warranting further research and development into applying CAP efficiently in long endoscope channels.

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Author contributions
RCH designed and carried out all plasma experiments, analysed and compiled results, and drafted the manuscript. MGK and SB developed the different prototypes tested and advised on experimental design. EC and J-PD performed the animal assay. TJS assisted with some plasma experiments and performed the cell assay. CWK helped with the design of the study and reviewed the manuscript.

Conflict of interest statement
None declared.

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