

# Cleaning of used rotary nickel-titanium files in an ultrasonic bath by locally intensified acoustic cavitation

L. M. Bryson<sup>1</sup>, D. Fernandez Rivas<sup>2</sup>  & C. Boutsoukis<sup>1</sup> 

<sup>1</sup>Department of Endodontology, Academic Centre for Dentistry Amsterdam (ACTA), Amsterdam; and <sup>2</sup>Mesoscale Chemical Systems Group, Faculty of Science and Technology, University of Twente, Enschede, The Netherlands

## Abstract

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**Aim** To compare the pre-sterilization cleaning of rotary Ni-Ti files of different sizes previously used a. *ex vivo* and b. clinically by a washer–disinfector, a regular ultrasonic bath, and the same ultrasonic bath in combination with a recently developed cavitation intensifying method.

**Methodology** Two sets of two hundred rotary Ni-Ti files, one previously used *ex vivo* and another one used clinically, were collected from the undergraduate and postgraduate clinics of the Academic Centre for Dentistry Amsterdam (ACTA). The instruments were immersed in an enzymatic solution and subsequently cleaned either by a washer–disinfector, a regular ultrasonic bath combined with a glass beaker, the same bath combined with a beaker lined with two cavitation intensifying sheets or with two standard plastic sheets.

The positive control consisted of used files that did not undergo any cleaning and the negative control included new unused files. The instruments were then stained to reveal remaining protein material and scored under a stereoscopic microscope. The results were analysed by nonparametric statistical tests ( $\alpha = 0.05$ ).

**Results** No significant difference was found between the combination of the ultrasonic bath and the regular glass beaker and the same ultrasonic bath with the beaker lined with the cavitation intensifying sheets. The washer–disinfector left significantly more debris compared to the latter group when clinically used files were evaluated ( $P \leq 0.001$ ). The effect of instrument size on cleaning was not consistent.

**Conclusion** None of the tested methods was able to remove all residual protein material from the files; however, it could be noted that this study did not follow the reprocessing protocol provided by the manufacturer.

**Keywords:** cavitation, cleaning, rotary instruments, ultrasonic bath, washer–disinfector.

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Correspondence: Dr. Christos Boutsoukis, Department of Endodontology, Academic Centre for Dentistry Amsterdam (ACTA), Gustav Mahlerlaan 3004, 1081 LA, Amsterdam, The Netherlands (Tel.: +31 20 59 80140; e-mail: c.boutsoukis@acta.nl).

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## Introduction

Rotary Nickel-Titanium (Ni-Ti) instruments are widely used for root canal preparation by both endodontists and general dentists (Parashos & Messer 2005, Bird *et al.* 2009, Locke *et al.* 2013). Owing primarily to their cost (Eggert *et al.* 1999), there is an interest in reusing these instruments on different patients to make treatments more affordable (Messer *et al.* 2003) provided that the instruments are not labelled as single use by the manufacturer. Reuse does not seem to

increase the risk of intracanal fracture (Gambarini 2001, Parashos *et al.* 2004a, Kosti *et al.* 2011), but it requires effective reprocessing of the instruments to eliminate the risk of cross-infection.

Apart from the well-recognized risk of patient-to-patient transmission of bacteria and viruses via contaminated instruments, there is worldwide interest over the risk of iatrogenic transmission of the Creutzfeldt-Jakob disease (CJD) and especially its variant form (vCJD) which is characterized by a wider distribution of the pathological prion protein within the human body (Head *et al.* 2003, Smith *et al.* 2003, Letters *et al.* 2005, Lipscomb *et al.* 2007, Azarpazhooh & Fillery 2008, Walker *et al.* 2008). Currently, there is no proven direct association between transmission of CJD (any form) and dentistry (Smith *et al.* 2003, Azarpazhooh & Fillery 2008), and there are no documented cases of patients that have been infected with CJD via used root canal instruments. In addition, pathological prion proteins have not been detected so far in the pulp tissue of patients diagnosed with vCJD (Head *et al.* 2003, World Health Organization 2010). Therefore, the risk of vCJD transmission during root canal treatment remains theoretical.

Nevertheless, concerns have been expressed about the small number of patients examined, the limited amount of pulp tissue that was tested and the sensitivity of the detection assays (Walker *et al.* 2008); prion proteins at levels below those detectable by these assays could still transfer the disease (Walker *et al.* 2009). Animal studies have detected low levels of prion infectivity in several oral tissues including pulp tissue (Walker *et al.* 2008) and have also demonstrated that the disease can be transmitted by exposing root canals to infectious material (Ingrosso *et al.* 1999, World Health Organization 2000), even though the models did not mimic very closely the possible human-to-human transmission through reused endodontic files and they completely ignored the use of NaOCl as an irrigant, which is known to have an effect against prion proteins (World Health Organization 2000). Despite the lack of evidence, the theoretical risk of vCJD transmission during root canal treatment via remaining biological material on reused files cannot be ruled out completely (Azarpazhooh & Fillery 2008, Walker *et al.* 2008, 2009), although it is not considered as a contradiction of file reuse following adequate reprocessing according to manufacturer recommendations or other validated protocols (Messer *et al.* 2003).

Reprocessing of used instruments involves several cleaning steps to remove tissue remnants or any

other organic residue that could interfere with sterilization (Johnson *et al.* 1997, Linsuwanont *et al.* 2004, Letters *et al.* 2005, Walker *et al.* 2009) and also to reduce the initial microbial load (Rutala & Weber 2001). Instruments are subsequently sterilized, most often in an autoclave (Linsuwanont *et al.* 2004). However, prion proteins are resistant to conventional chemical and thermal decontamination methods (Taylor 2000); drying may fix residual material on the instrument and render prions even more difficult to remove (Walker *et al.* 2008). Furthermore, prions bind firmly on stainless steel surfaces and remain infectious for extended periods of time (Zobley *et al.* 1999, Flechsig *et al.* 2001, Lipscomb *et al.* 2007); relevant information concerning Ni-Ti surfaces is lacking. To minimize the theoretical risk of iatrogenic CJD transmission during root canal treatment, the World Health Organization (WHO) has recommended that all dental instruments used for the treatment on known or suspected cases of CJD should not be reused on other patients, and even instruments used on healthy patients should be cleaned prior to sterilization to the highest possible standard (World Health Organization 2000).

Cleaning of root canal files is a challenging task due to their complex geometry (Smith *et al.* 2002, Walker *et al.* 2009). Most chemical methods recommended by WHO for this purpose (World Health Organization 2000) are potentially detrimental to metal surfaces (Smith *et al.* 2003) and, notably, corrosion of Ni-Ti files has been reported when they were applied (Sonntag & Peters 2007). In addition, they cannot achieve complete removal of organic debris from used rotary Ni-Ti files (Sonntag & Peters 2007). Ultrasonic baths combined with milder disinfectants are widely used instead for this purpose by dentists (Bagg *et al.* 2007), and even though they are also unable to remove all residual biological material consistently (Marening *et al.* 1998, Eggert *et al.* 1999, Letters *et al.* 2005, Smith *et al.* 2005), they are considered more effective than other available methods, such as hand scrubbing (Letters *et al.* 2005, Smith *et al.* 2005) and washer-disinfectors (Perakaki *et al.* 2007).

Efforts have been made to improve instrument cleaning by combining vigorous manual strokes in a sponge, pre-soaking in an enzymatic solution and ultrasonication (Parashos *et al.* 2003). This combined protocol has given promising results under both laboratory and clinical conditions (Linsuwanont *et al.* 2004, Parashos *et al.* 2004b), but it involves time-consuming manual actions and some reprocessed files

may still carry residual biological material. Further optimization of ultrasonic bath cleaning could improve the results, accelerate this procedure, and also reduce the necessary operator interventions and the related risk of percutaneous injuries.

Currently available ultrasonic baths dissipate the acoustic energy throughout the liquid volume instead of focusing its effect on the instruments to be cleaned. Moreover, the formation of cavitation bubbles is very unpredictable in both space and time and resembles a random process (Verhaagen *et al.* 2016). Cleaning of root canal files could be enhanced by introducing a novel sonochemical reactor into the ultrasonic bath which could intensify the cavitation locally in a well-defined manner (Verhaagen *et al.* 2016). This reactor has been recently realized in the form of a polypropylene bag (Cavitation Intensifying Bag, BuBclean, Borne, the Netherlands) (Fig. 1) having micropits onto its inner surface (diameter 100–500  $\mu\text{m}$ , depth 100–200  $\mu\text{m}$ ) that provide artificial nucleation sites for cavitation bubbles (Verhaagen *et al.* 2015, 2016) and it appears to be useful for emulsification and for improving the reproducibility of micron-sized object cleaning and exfoliation of nanomaterials (Gomes *et al.* 2017, van Zwieten *et al.* 2017). Nevertheless, the feasibility and effectiveness of such an approach

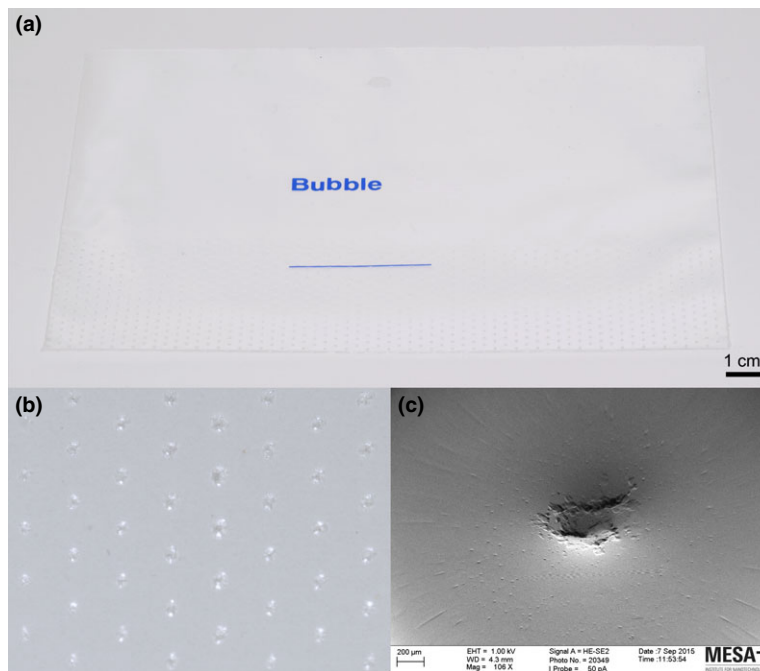
regarding cleaning of root canal files have not been tested.

Therefore, the aim of this study was to compare the pre-sterilization cleaning of rotary Ni-Ti files that had been previously used a. *ex vivo* and b. clinically by a washer–disinfector, an ultrasonic bath alone and an ultrasonic bath in combination with the Cavitation Intensifying Bag. The null hypothesis was that there is no significant difference between the compared cleaning methods.

## Materials and methods

### Part A. Cleaning of files used *ex vivo*

Two hundred used 25-mm rotary Ni-Ti files (Mtwo, VDW, Munich, Germany) of size 20, .06 taper ( $n = 100$ ) and size 35, .04 taper ( $n = 100$ ) were collected during the preclinical training of fourth-year undergraduate students at the Academic Centre for Dentistry Amsterdam (ACTA). Each file had been used by one student for the instrumentation of one extracted human maxillary or mandibular molar with either 3 or 4 root canals under simulated clinical conditions according to the preclinical training programme. No information was available about the age



**Figure 1** (a) The Cavitation Intensifying Bag. (b) Close-up view of the micro-pitted part. (c) Scanning Electron Microscopy photo of a micro-pit (x106).

of each tooth, the time and reason for extraction, the condition of the pulp before extraction, and the storage conditions, although students were advised to keep the teeth in distilled water until use. Teeth with very curved or obliterated root canals were excluded from the preclinical training.

All root canals were instrumented using new files according to the standard undergraduate protocol. Briefly, the coronal third of each root canal was scouted by a size 08 or 10 K-file (Dentsply Sirona, Ballaigues, Switzerland) and then enlarged by a size 20, .06 taper rotary Ni-Ti file (Mtwo, VDW). Subsequently, size 08, 10 and 15 K-files (Dentsply Sirona) were advanced to the full-working length and then size 10, .04 taper, 15, .05, 20, .06, 25, .06, 30, .05 and 35, .04 rotary Ni-Ti files (Mtwo, VDW) were used to the full-working length. Large straight root canals (mostly palatal canals of maxillary molars and distal canals of mandibular molars) were further enlarged to size 40, .04 taper. Instrumentation always took place in the presence of abundant 2% NaOCl solution in the root canal and pulp chamber and 1 mL of fresh irrigant was delivered in the root canal after each file using a 12-mL syringe and an open-ended needle (Navitip, Ultradent, South Jordan, UT, USA) placed 2 mm short of working length. No EDTA gel or liquid was used during instrumentation. During the session, the files were kept in a sponge soaked in 2% NaOCl.

This study did not follow the reprocessing protocol provided by the manufacturer of the rotary instruments (VDW 2017). The instruments were collected within 1 h after use, and they were kept in sealed hard-plastic containers filled with distilled water for a period up to six months. The distilled water was replaced every week. All instruments were briefly examined under a stereoscopic microscope (Stemi SV-6; Zeiss, Göttingen, Germany) at x40 magnification without drying to exclude fractured or deformed files. Special care was taken to grasp the files only from the shank using mosquito forceps and not to touch the cutting part in order to avoid further contamination; powder-free nitrile gloves were also used at all times. Rubber-stoppers were removed, and the files were randomly allocated ([www.randomizer.org](http://www.randomizer.org)) to five groups, each one including 20 files of size 20 and 20 files of size 35. Moreover, forty new and unused 25-mm rotary Ni-Ti files of size 20 and 35 (Mtwo, VDW) were used as negative controls.

All cleaning procedures were completed within the same day for each group according to the following protocols:

*Washer–disinfector (WD):* The files were first immersed in a 250-mL glass beaker (Schott Duran, Mainz, Germany) containing 150 mL of an enzymatic cleaning solution (HS-thermo, Henry Schein, Berlin, Germany) for 30 min (pre-soaking). Subsequently, the instruments were washed under running tap water for 20 s, arranged in two metal file holders (Endo top plus 32, Nichrominox, Saint-Bonnet-de-Mure, France) without any attempt to dry them, and they were immediately subjected to cleaning in a washer–disinfector (PG 8528, Miele Professional, Gütersloh, Germany) operating at the intensive cycle.

*Ultrasonic bath (US):* Pre-soaking of the instruments took place as in group WD. Next, the instruments were immersed in a 600-mL glass beaker (wall thickness = 1.85 mm, Schott Duran) containing 150 mL of the enzymatic solution, and the beaker was submerged in an ultrasonic bath with a nominal power of 130 W operating at a frequency of 40 kHz (3510, Branson, Danbury, CT, USA) and filled with tap water at room temperature up to the recommended level. The beaker was held in place above the left ultrasonic transducer by a metal clamp, and its vertical position was adjusted to optimize the transmission of pressure waves to the beaker based on maximum audible feedback. The bath was operated at 100% power for 15 min at 20°C. Only 20 instruments were placed at a time in the beaker ensuring that they were horizontal at the bottom and not overlapping with each other. At the end, the instruments were rinsed under running tap water for 20 s.

*Ultrasonic bath and cavitation intensifying sheets (US-CS):* Pre-soaking of the instruments took place as in group WD. Afterwards, the two sheets of a Cavitation Intensifying Bag were totally separated, cut to a circular shape exactly fitting a 600-mL glass beaker and placed horizontally at the bottom of the beaker. The instruments were placed in-between the two sheets ensuring that the pitted surface of each sheet was facing the instruments. Only 20 instruments were placed at a time in the beaker. The beaker was filled with 150 mL of the enzymatic solution, and then a second glass beaker (250-mL, wall thickness = 1.85 mm, Schott Duran) filled with tap water was placed on top of the upper bag sheet to act as a counter weight and prevent the bag from floating during ultrasonic cleaning. The same parameters were used during ultrasonication as in group US.

*Ultrasonic bath and standard plastic sheets (US-SS):* the same process was used as in group US-CS, but standard plastic bags (without micropits) were used

instead. This group was included as an additional control to clarify the effect of the micropits.

*Positive control (PC):* the used instruments in this group were not subjected to any cleaning or rinsing procedure.

*Negative control (NC):* the unused instruments in this group were not subjected to any cleaning or rinsing procedure.

Next, the instruments were stored in covered Petri dishes to minimize exposure to dust and left overnight to dry at room temperature. No air-blasts were used to dry them at any time. Coloured orthodontic rubber rings (Sani-Tie, Dentsply GAC International, Islandia, NY, USA) were fitted carefully to the shank of each instrument using mosquito forceps to facilitate tracking of individual instruments within each group. To evaluate the presence of protein residues, the instruments were next immersed in van Gieson's stain (1.2% picric acid and 1% acid aqueous fuchsine, Sigma-Aldrich, St. Louis, MO, USA) for 3 min and then rinsed with distilled water. This solution is used in histology for staining of collagen and other connective tissue proteins which appear red, orange or yellow (Linsuwanont *et al.* 2004, Sonntag & Peters 2007).

Subsequently, the instruments were examined under the stereoscopic microscope (Stemi SV-6, Zeiss) at 40× magnification to quantify the amount of remaining stained debris (red, orange, yellow). A custom-made holder was used to allow stable and reproducible positioning of the files under the microscope. The cutting part of each file was visually divided longitudinally in four sectors of equal length (4 mm) with the help of a ruler, and each part was evaluated at all four sides by rotating the holder block through 90°; this process allowed for evaluation of the entire cutting surface of each instrument. A 6-point scoring system was developed to quantify the amount of remaining debris (Table 1). A score was assigned to each side of each file sector, resulting in a total of 16 scores per file. When in doubt, a higher score was assigned. A single evaluator scored all files; the evaluator was initially calibrated through joint scoring of 20 additional used files (not included in the main experiments) together with a second evaluator. During scoring of the files used in the main experiments, the evaluator was blinded to the group that each file belonged to. The intra-observer agreement was evaluated by repeating the scoring process on a random 10% sample of all instruments 10 weeks after the first scoring.

**Table 1** The scoring system used to evaluate the amount of residual debris on the instruments

Score	Description
0	Sector completely clean, no visible debris.
1	Few scattered particles smaller than 0.05 mm, spaced apart, no continuous areas of debris.
2	Scattered particles larger than 0.05 mm, spaced apart, no continuous areas of debris.
3	Continuous areas of debris not extending more than 1 mm of length in total (<25% of sector).
4	Continuous areas of debris extending 1–2 mm of length in total (25–50% of sector).
5	Continuous areas of debris extending more than 2 mm of length in total (>50% of sector).

### Part B. Cleaning of files used clinically

Two hundred used rotary Ni-Ti files (Mtwo, VDW) of size 20, .06 taper ( $n = 100$ ) and size 35, .04 taper ( $n = 100$ ), both 25-mm and 21-mm in length (in equal numbers), were also collected from the postgraduate Endodontic clinic of the Academic Centre for Dentistry Amsterdam (ACTA). These files had been used by postgraduate students for patient treatment. No identifiers were attached to the files so there was no information about the identity or age of each patient, the type of tooth treated, the diagnosis or the number of root canals prepared. However, all instruments were discarded after use during a single session. Instruments used in retreatment cases were excluded to prevent confusion of gutta-percha remnants with stained biologic material.

The instrumentation protocol was not standardized in the postgraduate clinic and could vary depending on individual root canal anatomy. A minimum instrumentation size of 35 was recommended, but the students could combine instruments of different types and brands in customized protocols to reach this goal. Instrumentation always took place in the presence of abundant 2% NaOCl solution in the root canal and pulp chamber and fresh irrigant was copiously delivered in the root canal using a 12-mL syringe and an open-ended needle (Navitip, Ultradent) placed 2 mm short of working length. No EDTA gel or liquid was used during rotary instrumentation. During the treatment session, the files were kept in a sponge soaked in 2% NaOCl. At the end of the session, the files were discarded in clearly identified sealed hard-plastic containers filled with distilled water.

Handling, allocation to the five groups, cleaning and evaluation were identical to the files used *ex vivo* (Part A), but additional special care was taken to



prevent percutaneous injuries. Each group included 21-mm and 25-mm files in equal numbers. Forty new rotary Ni-Ti files of size 20 and 35 (Mtwo, VDW) were used as negative control.

**Statistical analysis**

The highest rather than the median of the 16 scores assigned to each instrument was selected to characterize it; this score was used in the statistical analysis. The scores in each group were compared by the nonparametric Kruskal–Wallis test and *post hoc* Dunn’s test, separately for instruments used *ex vivo* (part A) and clinically (part B) and for each size. The scores of size 20 and size 35 files were compared within each group by the nonparametric Mann–Whitney test under the null hypothesis of no significant difference. The alpha level was set at 0.05; Bonferroni

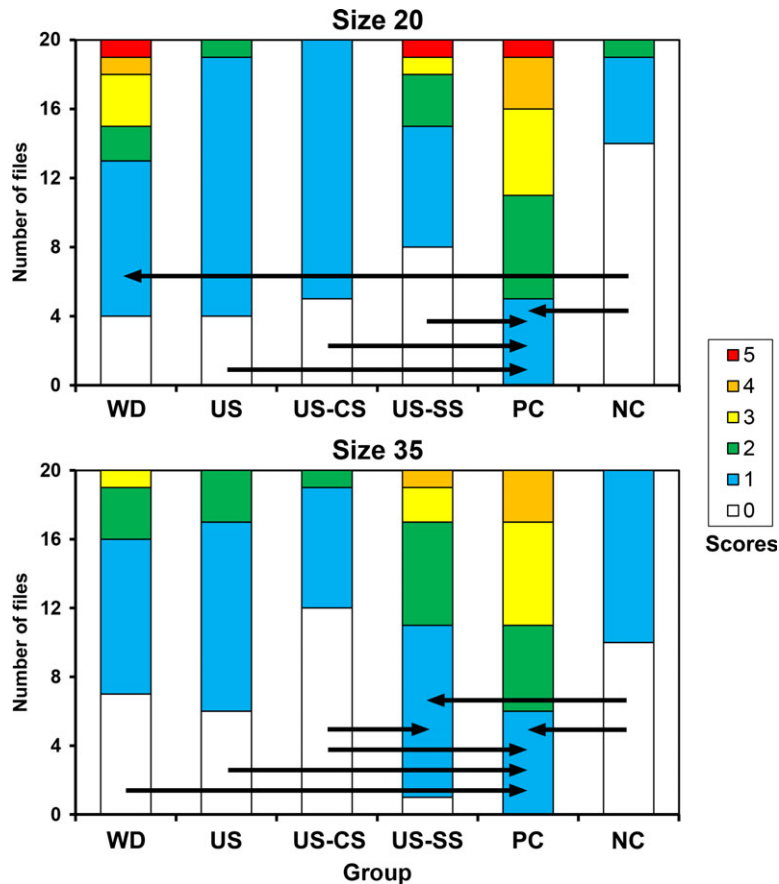
correction for multiple comparisons was applied to this level where appropriate. Effect size estimates (*r*) were calculated as the ratio of z-values to the square root of the combined sample size, according to Field (2009). The intra-observer agreement was evaluated by the weighted-kappa coefficient using quadratic weights. Statistical analysis was performed using SPSS Statistics 20.0 (IBM Corp, Armonk, NY, USA).

**Results**

The weighted-kappa coefficient was 0.86, which indicated an almost perfect intra-observer agreement.

**Part A. Cleaning of files used *ex vivo***

Seventy-one instruments were completely free of stained material (Fig. 2). The omnibus test indicated



**Figure 2** Scores of the amount of stained debris identified in each group during cleaning of the files used *ex vivo*, separately for size 20 and size 35 files. Arrows indicate statistically significant differences between groups, and they are pointing towards the group having the higher scores. WD: washer–disinfector, US: ultrasonic bath, US-CS: ultrasonic bath and cavitation intensifying sheets, US-SS: ultrasonic bath and standard plastic sheets, PC: positive control, NC: negative control.

**Table 2** *P*-values and effect size estimates (*r*) for the pairwise comparisons between the six groups regarding the removal of stained debris from size 20 files (upper right diagonal half, white cells) and size 35 files (bottom left diagonal half, grey cells) used *ex vivo*. The alpha level has been adjusted according to the Bonferroni method

	WD	US	US-CS	US-SS	PC	NC
WD	–	0.175	0.081	0.157	0.012	<b>&lt;0.001*</b> <b><i>r</i> = 0.56</b>
US	0.983	–	0.697	0.953	<b>&lt;0.001*</b> <b><i>r</i> = 0.61</b>	0.027
US-CS	0.098	0.102	–	0.741	<b>&lt;0.001*</b> <b><i>r</i> = 0.67</b>	0.068
US-SS	0.021	0.020	<b>&lt;0.001*</b> <b><i>r</i> = 0.63</b>	–	<b>&lt;0.001*</b> <b><i>r</i> = 0.62</b>	0.031
PC	<b>&lt;0.001*</b> <b><i>r</i> = 0.61</b>	<b>&lt;0.001*</b> <b><i>r</i> = 0.61</b>	<b>&lt;0.001*</b> <b><i>r</i> = 0.87</b>	0.126	–	<b>&lt;0.001*</b> <b><i>r</i> = 0.96</b>
NC	0.162	0.169	0.796	<b>&lt;0.001*</b> <b><i>r</i> = 0.59</b>	<b>&lt;0.001*</b> <b><i>r</i> = 0.83</b>	–

WD, washer–disinfector; US, ultrasonic bath; US-CS, ultrasonic bath and cavitation intensifying sheets; US-SS, ultrasonic bath and standard plastic sheets; PC, positive control; NC, negative control.

\*Statistically significant difference (bold).

a significant difference for both size 20 and size 35 files ( $P < 0.001$ ). Size 20 instruments in the positive control group had significantly ( $P < 0.001$ ) more debris compared to all other groups except the washer–disinfector group; the latter group had also significantly ( $P < 0.001$ ) more debris than the negative control group. Regarding the size 35 instruments, the positive control group had significantly ( $P < 0.001$ ) higher scores compared to all other groups except the group employing the ultrasonic bath and the standard plastic sheets; the latter group also had significantly ( $P < 0.001$ ) higher scores compared to both the group employing the ultrasonic bath and the cavitation intensifying sheets and to the negative control group (Table 2). Size 35 files had significantly more debris than size 20 files in the group combining the ultrasonic bath and the standard plastic sheets ( $P = 0.037$ ,  $r = 0.33$ ).

### Part B. Cleaning of files used clinically

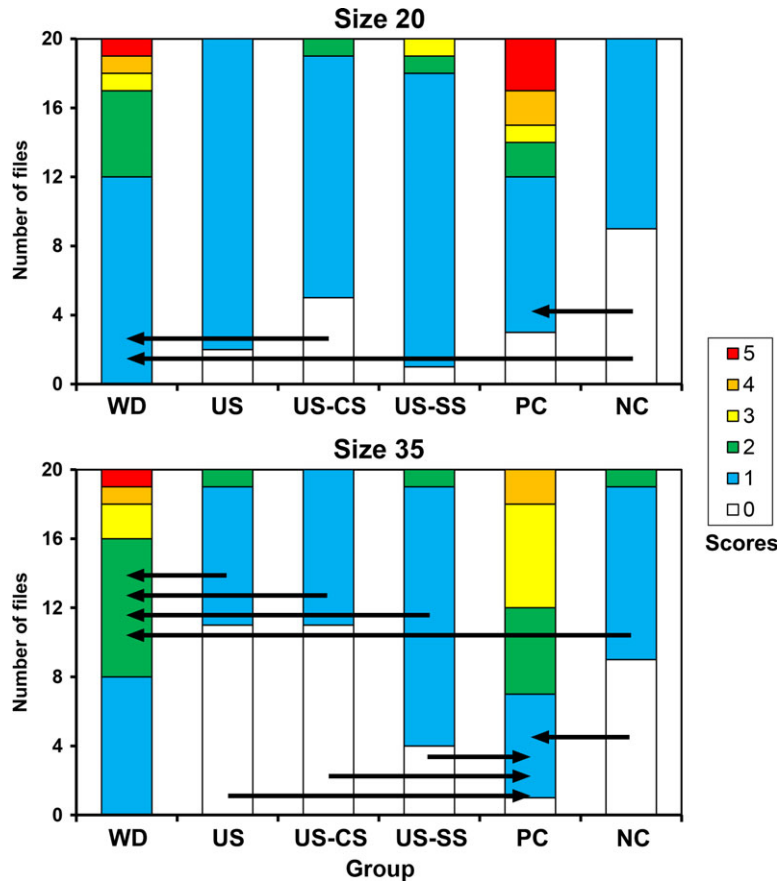
Fifty-six instruments were completely free of stained material (Fig. 3). The omnibus test also indicated a significant difference for both size 20 and size 35 files ( $P < 0.001$ ). The positive control and the washer–disinfector groups had significantly ( $P < 0.001$ ) higher scores than the negative control group concerning size 20 files. The washer–disinfector group also had significantly ( $P = 0.001$ ) higher scores than the group employing the ultrasonic bath and the cavitation intensifying sheets. Regarding size 35 files, all groups had significantly ( $P \leq 0.002$ ) less debris compared to

the positive control and the washer–disinfector groups, and there was no significant difference between the latter two groups (Table 3). Size 20 files had significantly more debris than size 35 files in the ultrasonic bath group ( $P = 0.006$ ,  $r = 0.42$ ).

### Discussion

Unnecessary discarding of rotary Ni-Ti files after a single use may have a serious impact on the overall cost of root canal treatment for both patients and dentists (Messer *et al.* 2003). Many types of root canal files, including the ones evaluated in the present study, are not strictly labelled as single use and reprocessing protocols are provided by the manufacturer (VDW 2017), even though single use may still be favoured in the directions for use (VDW 2015a,b). In addition, even new unused instruments may have debris and organic material (Linsuwanont *et al.* 2004, Parashos *et al.* 2004b, Sonntag & Peters 2007). Therefore, the search for more effective methods to clean and disinfect root canal files is pertinent. The present study aimed to evaluate a novel such method that could enhance cleaning in an ultrasonic bath by intensifying the production of cavitation bubbles near the files.

Both files used *ex vivo* and files used clinically were evaluated in the two parts of this study. The former files were easier to collect in larger numbers, and they had been used by less-experienced undergraduate students to prepare canals in extracted teeth according to a standardized protocol, so they possibly carried a



**Figure 3** Scores of the amount of stained debris identified in each group during cleaning of the files used clinically, separately for size 20 and size 35 files. Arrows indicate statistically significant differences between groups, and they are pointing towards the group having the higher scores. WD: washer–disinfector, US: ultrasonic bath, US-CS: ultrasonic bath and cavitation intensifying sheets, US-SS: ultrasonic bath and standard plastic sheets, PC: positive control, NC: negative control.

**Table 3** P-values and effect size estimates (*r*) for the pairwise comparisons between the six groups regarding the removal of stained debris from size 20 files (upper right diagonal half, white cells) and size 35 files (bottom left diagonal half, grey cells) used clinically. The alpha level has been adjusted according to the Bonferroni method

	WD	US	US-CS	US-SS	PC	NC
WD	–	0.007	<b>0.001*</b> <i>r</i> = 0.52	0.061	0.519	<b>&lt;0.001*</b> <i>r</i> = 0.73
US	<b>&lt;0.001*</b> <i>r</i> = 0.74	–	0.559	0.422	0.042	0.053
US-CS	<b>&lt;0.001*</b> <i>r</i> = 0.77	0.861	–	0.165	0.009	0.176
US-SS	<b>0.002*</b> <i>r</i> = 0.49	0.116	0.081	–	0.218	0.006
PC	0.861	<b>&lt;0.001*</b> <i>r</i> = 0.77	<b>&lt;0.001*</b> <i>r</i> = 0.80	<b>&lt;0.002</b> <i>r</i> = 0.52	–	<b>&lt;0.001*</b> <i>r</i> = 0.63
NC	<b>&lt;0.001*</b> <i>r</i> = 0.67	0.654	0.532	0.262	<b>&lt;0.001*</b> <i>r</i> = 0.70	–

WD, washer–disinfector; US, ultrasonic bath; US-CS, ultrasonic bath and cavitation intensifying sheets; US-SS, ultrasonic bath and standard plastic sheets; PC, positive control; NC, negative control.

\*Statistically significant difference (bold).



more uniform amount of debris. On the contrary, the files included in part B had been used clinically by more-experienced postgraduate students without adhering to a single protocol and probably carried a more realistic and varying amount of debris, so they better approximated real-life conditions in dental practices. Still, it must be emphasized that the collected files were kept in distilled water for extended periods of time before cleaning. This was inevitable due to the slow turnover of the rotary instruments especially in the postgraduate clinic, where a wide variety of instrument types and lengths are used and a large proportion of the cases involve retreatments. Even though keeping the instruments moist until cleaning was in accordance with current recommendations (World Health Organization 2000, Sonntag & Peters 2007), this long storage time may have affected the debris.

To accelerate the process and increase the number of clinically used files that could be included in part B, a pilot study was conducted to determine whether 25-mm and 21-mm files could be pooled. The results showed a significant difference between files of different length in some cases (data not shown), so equal numbers of 25-mm files and 21-mm files were finally included in each group in part B to balance the possible effect of file length. No specific attempt was made to compare 21-mm and 25-mm files in the main experiment due to the small sample size.

Van Gieson's solution has been used previously for staining of residual biologic material on files (Linsuwanont *et al.* 2004, Sonntag & Peters 2007), and it can easily stain a wide range of proteins (Linsuwanont *et al.* 2004). However, it cannot distinguish between pathological prion proteins and other kinds of proteins, so the stained material does not necessarily pose a risk of CJD transmission. In fact, van Gieson's staining was only employed as a preliminary step for initial assessment of the amount of residual protein on root canal instruments. More elaborate detection assays (Lipscomb *et al.* 2006) should be used in the future to verify these findings.

Several different scoring systems were used in previous studies to quantify the amount of remaining debris on files (Smith *et al.* 2002, Linsuwanont *et al.* 2004, Smith *et al.* 2005, Aasim *et al.* 2006, Perakaki *et al.* 2007, Sonntag & Peters 2007, Nosouhian *et al.* 2015). Nevertheless, these systems either lacked a clear definition of what is represented by each score (Linsuwanont *et al.* 2004, Sonntag & Peters 2007), contained too many steps (Smith *et al.* 2005,

Nosouhian *et al.* 2015) or required accurate visual estimation of the area covered by debris as a percentage of total area (Smith *et al.* 2002, Aasim *et al.* 2006, Perakaki *et al.* 2007, Nosouhian *et al.* 2015) which can be challenging. A new scoring system was developed by combining and modifying scores from Linsuwanont *et al.* (2004), Smith *et al.* (2005) and Sonntag & Peters (2007), in an effort to define a reasonable number of scores and to provide a clear definition of what each score represents. Visual aids were also used to facilitate the estimation of particle dimensions and of the affected surface area. This new scoring system resulted in almost perfect intra-observer agreement. Nonetheless, it did not allow for direct comparison of the results to earlier studies, and it was still a semiquantitative method prone to human error.

The experiments showed that the combination of an ultrasonic bath and the glass beaker lined with the cavitation intensifying sheets did not result in significantly improved cleaning compared to the ultrasonic bath alone regarding the removal of stained biological material. Taking into account that this cavitation intensifying method has been tested and found to provide advantages under different conditions (Verhaagen *et al.* 2016, Gomes *et al.* 2017, van Zwieten *et al.* 2017), it is possible that lining a glass beaker with the plastic sheets and placing a second beaker on top may have limited the performance of the micropits. Nevertheless, this application method was selected through a series of pilot tests (data not shown) and appeared to provide better results than placing each file into a separate bag and directly submerging them inside the ultrasonic bath. The latter method would also have very limited clinical relevance taking into account the workload of a dental practice. Another possible explanation is that a higher-power ultrasonic bath was necessary in order for the cavitation intensifying mechanism to be more effective. Finally, it is likely that the spacing between the artificial micropits from which cavitation is intensified (*ca.* 3.5 mm) may have been larger than necessary to affect the contaminated surface of the files. Moreover, root canal files are tapered, so the distance between the cavitation intensifying sheets and the file differed along the file, and this could have affected the production of cavitation bubbles. Nevertheless, the cavitation intensifying sheets generally gave better results than the standard plastic sheets, a trend that reached statistical significance in the case of size 35 files used *ex vivo*. It may be hypothesized that the presence of the plastic sheets may have reduced the cleaning efficacy of ultrasonication and

that micropits attenuated this negative effect to some extent and provided an advantage under these conditions. Future experiments should take into account these potential limitations.

It should be emphasized that ultrasonic bath cleaning is a sensitive procedure and several operating parameters can affect the result (Verhaagen & Fernandez Rivas 2016, Gomes *et al.* 2017). Ultrasonic transducers may degrade over time so their condition should be frequently checked. Furthermore, the bath should be filled with liquid up to the recommended level, and the instruments should be arranged in a way that allows cavitation bubbles to reach all surfaces. Overloading and poor arrangement of the instruments could limit the cleaning efficacy. These requirements were taken into account during the experiments but might be overlooked during real-life use.

Automated cleaning of root canal files in a washer-disinfector may reduce handling of the files and therefore the risk of percutaneous injuries (Assaf *et al.* 2008). However, the present results revealed that the washer-disinfector was clearly less effective than ultrasonication especially in the cleaning of files collected after clinical use. In addition, in several cases, the files cleaned in the washer-disinfector carried an amount of debris similar to the positive control group (files used but not cleaned at all); these findings were unexpected and at variance with earlier studies (Perakaki *et al.* 2007, Assaf *et al.* 2008). It has been reported that the type of file holder may affect the cleaning efficacy of washer-disinfectors (Assaf *et al.* 2008); the metal box used to hold the files in the present study was probably more suitable for this purpose than the holder used by Assaf *et al.* (2008) as it allowed water spray to penetrate inside it and reach the complete cutting part of the instruments. Nevertheless, it is still likely that storage in a basket could have allowed for more effective cleaning (Assaf *et al.* 2008), even though it would have also increased the risk of percutaneous injuries.

Earlier studies have reported conflicting findings regarding the effect of instrument size on the amount of remaining debris after cleaning. One study reported that the size of K-flex hand files had no effect on the amount of debris (Perakaki *et al.* 2007) whilst another study found that the size of Hedström files affected their cleaning (Van Eldik *et al.* 2004); the same study reported that the taper of rotary Profile GT Ni-Ti files did not have a significant effect. According to the results of the present study, differences between size 20 and size 35 instruments were only found in some

cases without any obvious pattern. However, it should be noted that the taper of the instruments was also different (.06 vs. .04) so these results may not be directly comparable to those of the earlier studies.

## Conclusion

No significant difference was found between the combination of the ultrasonic bath and the cavitation intensifying sheets and the ultrasonic bath alone regarding the amount of stained biologic material remaining on files that had been used either *ex vivo* or clinically. The washer-disinfector left significantly more debris compared to the latter group when clinically used files were evaluated. The effect of instrument size on cleaning was not consistent. None of the tested methods was able to remove all residual protein material from the instruments; however, it should be noted that the current study did not follow the reprocessing protocol provided by the manufacturer.

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## Conflict of interest

Dr. Fernandez Rivas reports nonfinancial support from BuBclean, outside the submitted work. In addition, Dr. Fernandez Rivas has a patent WO 2015/144918 pending. The other authors have stated explicitly that there are no conflicts of interest in connection with this article.

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