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Manuscript Region of Origin: Europe & Central Asia

Abstract: Introduction Purpose of this study was to assess the effects of NaOCl immersion and sterilization on the cyclic fatigue resistance of heat-treated NiTi rotary instruments.

Materials & Methods Two hundred-ten new 25/.06 Twisted Files and Hyflex CM were divided into 7 groups (n = 15) for each brand. Group 1 (control group) included new instruments that were not immersed in NaOCl neither subjected to autoclave sterilization. Groups 2 and 3 were composed of instruments dynamically immersed for 3 minutes in 5% NaOCl solution for 1 and 3 times respectively. Groups 4 and 5 consisted of instruments only autoclaved 1 and 3 times respectively. Groups 6 and 7 recruited instruments that received a cycle of both immersion in NaOCl and sterilization, 1 and 3 times respectively. Instruments were subsequently subjected to fatigue test. The surface morphology of fractured instruments was studied by field emission-scanning electron microscopy (FE-SEM) and x-ray energy-dispersive spectrometric (EDS) analyses. Means and standard deviations of number of cycles to failure (NCF) were calculated and statistically analyzed by two-way analysis of variance (P<.05).

Results Comparison among groups indicated no significant difference of NCF (P>.05) except for groups of Twisted Files sterilized 3 times without o with immersion in NaOCl (P<.05). HyFlex CM exhibited higher cyclic fatigue resistance than TF when files were sterilized 3 times, independently from the immersion in NaOCl (P<.05). EDS analysis showed the presence of an oxide-rich layer on the CM files external surface. No morphological or chemical differences were founded between files of the same brand subjected to different treatments.

Conclusions Repeated cycles of sterilization did not influence cyclic fatigue of NiTi files except for TF that demonstrated a significant decrease of flexural resistance after 3 cycles of sterilization. Immersion in NaOCl did not reduce significantly the cyclic fatigue resistance of all heat-treated NiTi files tested.

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Kenneth M. Hargreaves
Editor in Chief Journal of Endodontics

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23 Catania, 25.08.2017
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25

26 Dear Prof. Hargreaves,
27

28 I would like to submit to Journal of Endodontics our manuscript entitled :
29 **“Cyclic fatigue resistance of heat-treated nickel-titanium instruments after immersion in sodium**
30 **hypochlorite and/or sterilization”**
31

32 which I upload in accordance with journal requirements.
33

34
35 The paper is constructed as: “Original research”.
36

37 Many factors can influence the fatigue of NiTi instruments including immersion in sodium hypochlorite and
38 autoclave sterilization. Resistance to cyclic fatigue of NiTi rotary instruments can be increased by
39 improvements in the manufacturing process or by the use of heat-treated alloys with superior mechanical
40 properties. To date, there are no data in literature about the influence of immersion in sodium hypochlorite
41 and autoclave sterilization on heat-treated nickel-titanium instruments cyclic fatigue resistance. Thus, our
42 purpose “was to compare the cyclic fatigue resistance of heat-treated nickel-titanium instruments (HyFlex
43 CM and Twisted Files) after immersion in sodium hypochlorite and/or sterilization.”
44
45

46 Moreover, 5 Universities (Catania, “La Sapienza” Rome, Sacred Heart Rome and Modena-Reggio Emilia
47 from Italy, as well as Universidad Científica del Sur, Lima, Perù) were involved in this project and FE-
48 SEM and EDS analyses were added to investigate the surface of the heat-treated instruments after immersion
49 in sodium hypochlorite and/or autoclave sterilization.
50

51
52 Neither this work –which has been approved by all co-authors- nor any part of its essential substance, tables
53 or figures have been or will be published or submitted to another scientific journal or is being considered for
54 publication elsewhere.
55

56 Best regards,
57

58 **Ernesto Rapisarda**
59 Head I Dental Clinic, University of Catania
60 Ordinary Professor of Endodontic and Restorative Dentistry,
61 University of Catania
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Title Page

Cyclic Fatigue Resistance of Heat-Treated Nickel-Titanium Instruments After Immersion in Sodium Hypochlorite and/or Sterilization.

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Key Words

Autoclave sterilization, cyclic fatigue, heat-treated files, sodium hypochlorite, CM-wire, R-Phase

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The authors deny any conflicts of interest

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Author point by point response

Manuscript ID: **JOE 17-879**

Manuscript Title: **Cyclic Fatigue Resistance of Heat-Treated Nickel-Titanium Instruments After Immersion in Sodium Hypochlorite and/or Sterilization**

Dear Prof. Ken Hargreaves -Editor-in-Chief- Journal of Endodontics,

I would like to thank You for the opportunity to review the manuscript mentioned above.

All the corrections suggested by the Reviewers were performed.

A point-by-point response to the reviewers' comments was created and submitted with the revised manuscript, as well as, all changes in the revised manuscript were highlighted by underlining the changes as requested by the Reviewers.

Moreover, FE-SEM and EDS analyses were added in the revised manuscript to investigate the surface of the heat-treated NiTi endodontic instruments after immersion in sodium hypochlorite and/or autoclave sterilization.

We do hope that this manuscript is now acceptable for publication.

Best regards

Eugenio Pedullà

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Comments to Reviewers

Reviewer #1: The present study compares the cyclic fatigue resistance of two heat-treated NiTi instruments, Twisted Files and Hyflex CM, after immersion in NaOCl and/or autoclave sterilization. Since NaOCl immersion and autoclave sterilization are frequently applied in root canal irrigation and instrument disinfection, and thus exerting possible effects on the cyclic fatigue resistance of these two heat-treated instruments, this in vitro study does shed some light on clinical significance.

RESPONSE: We would like to thank the Reviewer #1 for the positive revision.

Still, the paper needs some amendments. Some syntax and editorial errors need to be removed. Also, paragraphs can be rearranged in a reasonable way, into longer ones for easier reading.

RESPONSE: We would thank the Reviewer #1 for the helpful comments. A syntax revision and paragraphs rearrange were conducted as suggested. We hope that now it is more readable.

The introduction covers the information about the two heat-treated NiTi files and infers the possible effects of autoclave sterilization and NaOCl contact on cyclic fatigue resistance, with solid evidence and clear structure.

RESPONSE: Thanks for the positive revision.

1
2 *Materials and methods are generally adequately described; however, more details of the artificial*
3 *stainless steel canal should be given, especially the size, length and taper. If SEM test could be*
4 *applied to observe the fracture surface, the inference of possible fracture mechanism would be*
5 *more powerful.*
6

7
8 RESPONSE: Thanks to the Reviewer #1 for the really helpful comments. A more detailed
9 description of the artificial stainless steel canal was performed in the Materials and Methods
10 (Paragraph 4th) as suggested:
11

12 It consists of a 36.8 mm x 25.4 mm x 9.5 mm metal block with a suitable artificial canal with 60°
13 angle of curvature and a 5-mm radius of curvature to the centre of the 1.5-mm wide canal. Radius
14 was measured to the central axis of the curvature according to the method of Schneider (23). The
15 centre of the curvature was 5 mm from the tip of the instrument. The apparatus enabled the
16 instrument to rotate freely within a stainless steel artificial canal at a constant (7, 23, 24).
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20 Moreover, FE-SEM and EDS analyses on fractured fragments were performed in order to
21 investigate the surface of the heat-treated instruments after immersion in sodium hypochlorite
22 and/or autoclave sterilization. In the Material and Methods section, you can read as follows:
23

24 The broken fragments were analyzed using a field emission-scanning electron microscope (FE-
25 SEM: Nova NanoSEM 450; FEI, Eindhoven, the Netherlands), using 600X magnification to
26 investigate the morphology of the fracture surface and 500X for the lateral views. This
27 magnification was chosen to disclose the main feature morphology but maintaining a large field of
28 view.
29

30 The chemical composition of the files surface and eventual debris identified by imaging was
31 obtained by FE-SEM equipped with a Si-drift detector for energy-dispersive X-ray spectrometry
32 (EDS: Quantax-200; Bruker, Berlin, Germany). EDS spectra were collected from the middle region
33 of each fractured specimen on 2000X magnified images.
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37 *The discussion provides some analysis of methods and results, but explanation of the points below*
38 *should be strengthened.*

39 *1) NaOCl immersion: why set 3 min as the working time? Maybe the immersion time could also*
40 *be considered one factor of no reduction in cyclic fatigue resistance. Besides, in page 9 line 41*
41 *'static' should read 'dynamic'.*
42
43

44 RESPONSE: Thanks for your comments. 3 minutes was chosen to better simulate clinical practice
45 as stated in the Discussion (Paragraph 3th).

46 Moreover, “static” has been replaced with “dynamic” as suggested, in the 10th Paragraph of
47 Discussion.
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49
50 *When listing different results between the present study and previous ones, differences are vaguely*
51 *attributed to different instrument size, different sterilization protocol, different cyclic fatigue test,*
52 *different methodology, different type of analysis, and so on. Could you please state the reasons of*
53 *performing differently from those studies? Are the experimental conditions in the present study*
54 *more pertinent to clinical situations?*
55 *e.g. different sterilization protocol: (ref 9) 135°C 32.5min; (ref 15&29) 134°C 35min; (ref 16)*
56 *132°C 6min; different cyclic fatigue test: (ref 16) 5mm radius 90° curve; (ref 29) 3mm radius 60°*
57 *curve; (ref 35) 3mm radius 45° curve.*
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RESPONSE: Thank You for the interesting comment. The sterilized instruments were subjected to 1 or 3 cycles of autoclave sterilization and each cycle was performed at a temperature of 134°C for 17 minutes. The differences with other studies depend on the type of sterilizer used and consequently the setting programs followed. Moreover, our parameters ensure a better simulation of clinical conditions, maintaining a correct protocol for an efficient disinfection (“Sterilization dental. Available at: <https://www.isopharm.co.uk/dental/sterilisation>, “Sterilix 2 Plus, brochure. Available at: <http://www.krescendomultimedia.it/sviluppo/reverberisrl/wp-content/uploads/2014/04/Vacuum-Star-GB-R09.pdf>”. Accessed November 16, 2017).

In addition, an artificial stainless steel canal with a 60° degree angle of curvature and 5 mm radius was used in this study. An ideal model would involve instrumentation of curved canals in natural teeth. However, in such tests a tooth can be used only once and the trajectory of the root canal changes during instrumentation, making it impossible to standardize experimental conditions (Plotino G, Grande NM, Cordaro M, Testarelli L, Gambarini G. A review of cyclic fatigue testing of nickel-titanium rotary instruments. J Endod 2009 Nov;35:1469-76). Therefore, we used this device with these specific parameters to reach standardization and a good canal anatomy reproduction (“Carrotte P. Endodontics Part 4: Morphology of the root canal system. British Dental Journal 2004; 197: 379-383). In fact, increased severity of angle and radius of the curve, around which the instrument rotates, decreases instrument life span in vitro and clinically, reproducing only a small percentage of clinical conditions. Consequently, these parameters (60° angle of curvature and 5 mm radius) are the most used in the previous studies on cyclic fatigue.

We synthetized these concepts in the Discussion (4th Paragraph), you can read as follows:

“An artificial canal with 60° angle of curvature and a 5-mm radius of curvature was chosen because the most of previous studies on cyclic fatigue were based on these parameters (13, 21, 26). Each cycle of autoclave sterilization was performed at a temperature of 134°C for 17 minutes. This temperature and this sterilization time ensure a better simulation of clinical conditions, maintaining a correct protocol for an efficient disinfection (33)”

Reviewer #3: Manuscript Number: JOE 17-879

The manuscript titled with "Cyclic Fatigue Resistance of Heat-Treated Nickel-Titanium Instruments After Immersion in Sodium Hypochlorite and/or Sterilization" investigated the influence of autoclave sterilization and NaOCl immersion on the cyclic fatigue of two heat treated NiTi files. It was clinic relevant. However, there were some suggestions as follows:

1. *In Introduction section, the scientific evidence to investigate the influence of autoclave on the fatigue property of Ni-Ti file was not strong.*

RESPONSE: Thanks to the Reviewer for the positive comments. Scientific background of the autoclave influence on the cyclic fatigue of NiTi rotary instruments has been further investigated in the Introduction (Paragraph 4th and 5th), as suggested:

Sterilization of NiTi files must be ensured before clinical use, except for the pre-sterilized ones. Repetitive use of files under clinical conditions requires the autoclave sterilization after every use. Also, pre-arranged sets of selected files may not be used during the same appointment. As a result, the unused rotary files are could also subjected to multiple autoclave cycles (13, 14).

Researchers reported that the additional heat treatment during autoclave sterilization might improve the flexibility of files, and the sterilization of files by using dry-hot air and autoclave would have positive effect on the cyclic fatigue resistance (13, 14).

1 Although no effect of autoclave sterilization on Lightspeed NiTi rotary instruments (Discus Dental,
2 Culver City, CA) has been reported; Profile NiTi rotary files have shown a higher mean cycle to
3 failure when exposed to both dry heat and autoclave sterilization (14, 15). Similar findings of
4 autoclave conditions either improving or degrading both the performance and physical properties of
5 various marketed rotary NiTi systems have been reported (9, 14-17).
6

7 *2. Although in this study TF showed a decreased of flexural resistance after 3 cycles of*
8 *sterilization, other studies reported opposite results. Why? How to explain?*
9

10 RESPONSE: Recently, Zhao et al (2016) reported that TF showed no difference in cyclic fatigue
11 resistance after autoclave sterilization. The different results with our study may be explained by
12 different instrument size, sterilization condition and methodology used. In addition, Bulem et al
13 (2013) shown as five repeated cycles of NaOCl and autoclave sterilization had no effect on cyclic
14 fatigue of TF. The different results with our study could be explained by the different methodology
15 performed, you can read as follow in the 7th Paragraph of the Discussion section:
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19 “The different results with our study could be explained by the different cyclic fatigue test
20 performed as well as the other experimental conditions (NaOCl concentration and time of
21 immersion used) (9).”
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Cyclic fatigue resistance of heat-treated nickel-titanium instruments after immersion in sodium hypochlorite and/or sterilization

Highlights

- Hyflex CM cyclic fatigue was not affected by NaOCl and/or sterilization.
- Three cycles of sterilization reduced Twisted Files cyclic fatigue resistance.
- The cyclic fatigue resistance of both instruments was not influenced by NaOCl.
- Hyflex CM had higher fatigue resistance than TF after three sterilization cycles.
- EDS analysis showed the presence of an oxide-rich layer on the CM files surface.

Significance

Immersion in sodium hypochlorite as well as autoclave sterilization could influence cyclic fatigue resistance of NiTi instruments. It is important to know as these procedures influence flexural fatigue of heat-treated NiTi files.

Cyclic Fatigue Resistance of Heat-Treated Nickel-Titanium Instruments After Immersion in Sodium Hypochlorite and/or Sterilization

Abstract

Introduction Purpose of this study was to assess the effects of NaOCl immersion and sterilization on the cyclic fatigue resistance of heat-treated NiTi rotary instruments.

Materials & Methods Two hundred-ten new 25/.06 Twisted Files and Hyflex CM were divided into 7 groups (n = 15) for each brand. Group 1 (control group) included new instruments that were not immersed in NaOCl neither subjected to autoclave sterilization. Groups 2 and 3 were composed of instruments dynamically immersed for 3 minutes in 5% NaOCl solution for 1 and 3 times respectively. Groups 4 and 5 consisted of instruments only autoclaved 1 and 3 times respectively. Groups 6 and 7 recruited instruments that received a cycle of both immersion in NaOCl and sterilization, 1 and 3 times respectively. Instruments were subsequently subjected to fatigue test. The surface morphology of fractured instruments was studied by field emission-scanning electron microscopy (FE-SEM) and x-ray energy-dispersive spectrometric (EDS) analyses. Means and standard deviations of number of cycles to failure (NCF) were calculated and statistically analyzed by two-way analysis of variance (P<.05).

Results Comparison among groups indicated no significant difference of NCF (P>.05) except for groups of Twisted Files sterilized 3 times without or with immersion in NaOCl (P<.05). HyFlex CM exhibited higher cyclic fatigue resistance than TF when files were sterilized 3 times, independently from the immersion in NaOCl (P<.05). EDS analysis showed the presence of an oxide-rich layer on the CM files external surface. No morphological or chemical differences were founded between files of the same brand subjected to different treatments.

Conclusions Repeated cycles of sterilization did not influence cyclic fatigue of NiTi files except for TF that demonstrated a significant decrease of flexural resistance after 3 cycles of sterilization. Immersion in NaOCl did not reduce significantly the cyclic fatigue resistance of all heat-treated NiTi files tested.

Introduction

1
2 Root canal instruments manufactured from nickel-titanium (NiTi) alloy were introduced in 1988 to
3 overcome the rigidity of stainless steel material (1, 2).
4

5
6 However, there is a general perception that NiTi instruments have a high risk of fracture during
7 their use (3) and manufacturers have continued efforts to improve the properties and fracture
8 resistance of NiTi instruments. Separation of NiTi files can be due to torsional or flexural fatigue
9 (cyclic fatigue) (4, 5, 6).
10

11
12 Many factors can influence the fatigue of NiTi instruments as type of kinematics (7), as well as raw
13 materials and manufacturing processes (8). Resistance to cyclic fatigue of NiTi rotary instruments
14 can be increased by improvements in the manufacturing process or by the use of heat-treated alloys
15 with superior mechanical properties (9).
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19 The Twisted file (TF) (Sybron Endo, Orange, CA, USA) is a NiTi rotary system manufactured with
20 R-phase alloy using a twisting method. Its manufacturing process involves the transformation of a
21 basic austenite NiTi wire into the R (pre-martensitic)-phase by a process of heating and cooling.
22 After the twisted shape is achieved, a series of heating and cooling is said to convert the twisted R-
23 phase wire back to the austenite crystalline structure, which becomes super-elastic while stressed
24 (10). This treatment would impart to the files an increased cyclic fatigue resistance (11).
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28 Another development in fabrication of endodontic NiTi instruments was the introduction of
29 controlled memory wire (CM Wire, DS Dental, Johnson City, TN) as Hyflex CM (Coltene
30 Whaledent, Cuyahoga Falls, OH, USA). These instruments have high resistance to cyclic fatigue
31 and do not rebound to their original shape, thanks to their alloy and specific manufacturing process
32 (11, 12).
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36 Because post-machining thermal treatment influences the properties of heat-treated alloys, also the
37 heat generating by sterilization procedures could influence the mechanical properties of endodontic
38 instruments (9, 13). Sterilization of NiTi files must be ensured before clinical use, except for the
39 pre-sterilized ones. Repetitive use of files under clinical conditions requires the autoclave
40 sterilization after every use. Also, pre-arranged sets of selected files may not be used during the
41 same appointment. As a result, the unused rotary files are could also subjected to multiple autoclave
42 cycles (13, 14). Researchers reported that the additional “heat-treatment” during autoclave
43 sterilization might improve the flexibility of files, and the sterilization of files by using dry-hot air
44 and autoclave would have positive effect on the cyclic fatigue resistance (13, 14).
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48 Although no effect of autoclave sterilization on Lightspeed NiTi rotary instruments (Discus Dental,
49 Culver City, CA) has been reported; Profile NiTi rotary files have shown a higher mean cycle to
50 failure when exposed to both dry heat and autoclave sterilization (14, 15). Similar findings of
51 autoclave conditions either improving or degrading both the performance and physical properties of
52 various marketed rotary NiTi systems have been reported (9, 14-17).
53

54
55 One additional factor potentially limiting the NiTi files resistance to fatigue fracture is corrosion
56 that may occur in the presence of sodium hypochlorite (NaOCl) solution (18). NaOCl is used as
57 irrigant and it selectively removes nickel from the instrument surface and causes micropitting (19),
58 negatively affecting physical and mechanical properties of NiTi files (20).
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1 Even if the effects of NaOCl contact or autoclave sterilization on cyclic fatigue of NiTi instruments
2 have been separately investigated (14-18, 21), few studies analyzed the influence of both autoclave
3 sterilization and NaOCl immersion on the cyclic fatigue of NiTi endodontic files (9, 22, 23).

4
5 Moreover, no studies have investigated the influence of autoclave sterilization and immersion in
6 NaOCl of instruments made by different heat-treated alloys such as Twisted file and Hyflex CM.

7
8 Thus, the purpose of this study was to investigate the effect of both NaOCl contact and repeated
9 autoclave cycles on the cyclic fatigue resistance of R-phase and CM wire files.
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Materials and Methods

Sample size estimation was calculated a priori with G*Power 3.1.9.2 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany).

A total of 210 new 25/.06 Twisted Files and Hyflex CM were selected for the test. All instruments had been previously inspected using a measuring stereomicroscope (SZR-10; Optika, Ponteranica – Bergamo - Italy) for any signs of visible deformation. None was discarded.

Instruments all from the same production lot, were randomly assigned into 7 groups (n = 15) for each brand. Group 1 (the control group) included new instruments that were not immersed in NaOCl neither subjected to autoclave sterilization. Groups 2 and 3 were composed of instruments dynamically immersed for 3 minutes in 5% NaOCl solution (Nicolor, OGNA Laboratory, Milan, Italy) at 37°C for 1 and 3 times respectively. All files were placed in small separate glass containers with the amount of the NaOCl solution necessary to contact 16 mm of the instruments length; dynamic immersion was allowed activating the endodontic instruments with a 6:1 reduction handpiece (Sirona Dental Systems GmbH, Bensheim, Germany), powered by VDW Silver Reciproc motor at 500 rpm. Immediately after removal from the solutions, all files were rinsed with bidistilled water to neutralize the effect of NaOCl, dried, registered with an ID number and stored in glass vials. Groups 4 and 5 consisted of instruments only autoclaved 1 and 3 times respectively. The remaining groups (6 and 7) recruited instruments that received a cycle of both immersion in NaOCl (as above described) and sterilization, 1 and 3 times respectively.

The sterilized instruments were subjected to 1 or 3 cycles of autoclave sterilization (Sterilix Vacuum Plus; Reverberi, Barco, Italy), and each cycle was performed at a temperature of 134°C for 17 minutes (9). Each file was placed in a separate endodontic sponge and packaged singularly for sterilization in pouches. Instruments that underwent multiple autoclave cycles were allowed to cool to room temperature after sterilization. Sponges were removed from sterilization packaging and re-packaged singularly in pouches before the subsequent autoclave cycles. In group 4 and 5 no additional cleaning or surface treatment procedures were performed on the files before, during, or after sterilization. In group 6 and 7 immersion in NaOCl was carried out before autoclaving.

Instruments of all groups of each brand were then subjected to cyclic fatigue testing using an artificial stainless steel canal. It consists of a 36.8 mm x 25.4 mm x 9.5 mm metal block with a suitable artificial canal with 60° angle of curvature and a 5-mm radius of curvature to the center of the 1.5-mm wide canal. Radius was measured to the central axis of the curvature according to the method of Schneider (24). The center of the curvature was 5 mm from the tip of the instrument. The apparatus enabled the instrument to rotate freely within a stainless steel artificial canal at a constant pressure (7, 24, 25). The apparatus was connected to the same 6:1 reduction handpiece and motor used for the dynamic immersion.

Cyclic fatigue tests were performed rotating the instruments in continuous rotation at 500 rpm (as suggested by the manufacturers). Torque was set at maximum level. The canals were covered with glass to prevent the instruments from slipping out (26). To reduce friction between the instrument and the metal canal walls, a special high-flow synthetic oil designed for lubrication of mechanical parts (Super Oil; Singer Co Ltd, Elizabethport, NJ, USA) was applied.

1 All instruments were rotated until fracture occurred; timing was stopped as fracture was detected
2 visually and/or audibly. To obviate human error, video recording was carried out simultaneously,
3 and the recordings were observed to cross-check the time of file separation.

4
5 The number of cycles to failure (NCF) for each instrument was calculated by multiplying the
6 number of rotations per the effective seconds of continuous rotation required for fracture. The
7 length of the fractured file tip was measured by using a digital microcaliper (Mitutoyo Italiana srl,
8 Linate (Milan), Italy).

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11 Data were subjected to the Shapiro-Wilk test to characterize their normality and statistically
12 analyzed using the two-way analyses of variance (ANOVA) and Tukey's multiple comparison *post-*
13 *hoc* test to assess significant differences between groups ($P < .05$).

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16 The broken fragments were analyzed using a field emission-scanning electron microscope (FE-
17 SEM: Nova NanoSEM 450; FEI, Eindhoven, the Netherlands), using 600X magnification to
18 investigate the morphology of the fracture surface and 500X for the lateral views. This
19 magnification was chosen to disclose the main feature morphology but maintaining a large field of
20 view.

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26 The chemical composition of the files surface and eventual debris identified by imaging was
27 obtained by FE-SEM equipped with a Si-drift detector for energy-dispersive X-ray spectrometry
28 (EDS: Quantax-200; Bruker, Berlin, Germany). EDS spectra were collected from the middle region
29 of each fractured specimen on 2000X magnified images.

30 31 32 33 34 35 36 37 **Results**

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39
40 Means and standard deviations of NCF after cyclic fatigue test for all groups of investigated
41 instruments were presented in **Table 1**.

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43 The inferential analysis revealed statistically significant differences between the different groups
44 considering the type of instrument as the independent variable (two-way ANOVA, $P < .05$,
45 interaction $< .05$). Moreover, there were statistically significant differences between the instruments
46 tested considering the type of treatment as the independent variable (two-way ANOVA, $P < .05$;
47 interaction $< .05$).

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51 Three minutes immersion in 5% NaOCl both for 1 or 3 times did not exert any significant overall
52 effect on file performance for any of the instrument systems tested ($P > .05$).

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55 Twisted Files autoclaved 3 times had significantly lower resistance to cyclic fatigue than new ones
56 independently to the immersion in NaOCl ($P < .05$). Instead, cyclic fatigue of TF was not adversely
57 affected by sterilization procedure for 1 time, with or without the contact with NaOCl ($P > .05$). On
58 the other hand, no cyclic fatigue difference was found between the control and all test groups of
59 Hyflex CM ($P > .05$).

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1 Post-hoc analysis revealed a significantly higher cyclic fatigue resistance of HyFlex CM than TF
2 only in the groups 5 and 7 (files sterilized 3 times without or with immersion in NaOCl respectively)
3 ($P < .01$). Lengths of the fractured file fragments were not statistically different among the tested
4 instruments (mean = 5.1 mm) ($P > .05$) (**Table 1**).
5

6 The FE-SEM analysis of the fractured surfaces showed the typical features of cyclic fatigue failure
7 for both instruments. Crack initiation areas and overload fast fracture zone due to cyclic fatigue are
8 identifiable in the fracture surface of all instruments **Figure 1 (A-F)**. HyFlex CM files showed
9 surfaces grooves approximately orthogonal to their longitudinal axes (Fig. 2A), while surfaces grooves
10 approximately parallel to the longitudinal axes were founded on the Twisted Files (Fig. 2D).
11

12 The EDS analysis indicated that both tested instruments (Hyflex CM and TF) had near equiatomic
13 NiTi compositions (Fig. 2C, Sp. 3 and 2F, Sp. 3). Debris were visible on files surfaces of Hyflex
14 CM (Fig. 2C, Sp. 1) and Twisted Files (Fig. 2F, Sp. 1). Moreover, EDS analysis showed the
15 presence of an oxide-rich layer on the CM files external surface (Fig. 2C, Sp. 3) while the
16 underlying substrate was consisted of NiTi alloy with only a tiny amount of oxygen (Fig. 2C, Sp.
17 2).
18

30 Discussion

31 NiTi instruments are frequently re-used for multiple cases leading to an accumulation of cyclic
32 fatigue. Material fatigue appears to be an important reason for the separation of rotary instruments
33 during clinical use (27, 28).
34

35 The choice to reuse NiTi files for more than one patient makes it essential the sterilization process
36 in order to prevent cross-infections (29). Several studies had evaluated the effects of sterilization on
37 the mechanical properties of files manufactured with conventional NiTi alloy (14, 15), sometimes
38 resulting in conflicting results. Although some authors reported no effect of autoclave sterilization
39 on fatigue resistance of NiTi files (9, 15), others have shown a higher (14, 16) or lower (17) mean
40 NCF when exposed to both dry heat and autoclave sterilization. In addition, few reports investigated
41 the influence of autoclave sterilization procedure on cyclic fatigue of heat-treated NiTi instruments
42 (9, 16, 17, 30) and only one involved also the immersion in sodium hypochlorite (9).
43

44 Thus, in the present study, we enquired into the effect of both NaOCl contact and autoclave
45 sterilization on the cyclic fatigue resistance of two heat-treated NiTi files. Present investigation was
46 based on rotary endodontic instruments that had not been used clinically in order to avoid the
47 instrument exposure to the uncontrolled stresses of routine clinical conditions, like in previous
48 reports (14, 15). In order to get closer to clinical conditions, the protocol of this study provides
49 dynamic immersion in 5% NaOCl solution at 37°C for 3 minutes only of the working part (16 mm)
50 (21). Only 16 mm from the tip of instruments was immersed in solution to avoid galvanic corrosion
51 phenomena (31). To simulate a realistic number of times that files can be reused, it was chosen a
52 number of 1 or 3 cycles of sterilization and or immersion in sodium hypochlorite (32).
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1 An artificial canal with 60° angle of curvature and a 5-mm radius of curvature was chosen because
2 the most of previous studies on cyclic fatigue were based on these parameters (13, 21, 26). Each
3 cycle of autoclave sterilization was performed at a temperature of 134°C for 17 minutes. These
4 temperature and sterilization time ensure a better simulation of clinical conditions, maintaining a
5 correct protocol for an efficient disinfection (33).
6

7 Under the present conditions, a statistically significant decrease in TF cycles to failure after
8 autoclaving for 3 times was observed.
9

10 The current results suggest any enthalpy generated during autoclave processing does not provide
11 enough energy to enable a heat-treatment effect that could cause a crystalline phase change. It has
12 been reported that a temperature of 170° C is required to initiate reordering, 430°C to 440°C to
13 obtain maximum fatigue resistance (15). Furthermore, multiple autoclave cycles have been reported
14 to increase the depth of NiTi file surface irregularities, causing fatigue propagation (34, 35).
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19 Even if the present report is in agreement with a previous study (17) in which autoclaving
20 procedures significantly reduced the NCF of 25/.06 Twisted Files instruments, it is in contrast with
21 another one in which repeated cycles of autoclave sterilization do not affect mechanical properties
22 of NiTi endodontic instruments except for the K3 XF prototypes rotary instruments that
23 demonstrated a significant increase of their cyclic fatigue resistance. (16). These different findings
24 could be due to the different instrument tested as well as the sterilization protocol followed (16).
25 Only one study analyzed the effect of NaOCl immersion and autoclave sterilization on cyclic
26 fatigue of heat-treated NiTi alloys (9), excluding any influence on cyclic fatigue resistance when TF
27 are subjected to five repeated cycles of NaOCl and autoclave sterilization. The different results with
28 our study could be explained by the different cyclic fatigue test performed as well as the other
29 experimental conditions (NaOCl concentration and time of immersion used)(9).
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36 Regarding Hyflex CM, autoclave sterilization conditions did not significantly affect its cyclic
37 fatigue behavior. In addition, HyFlex CM exhibited higher cyclic fatigue resistance than TF, only in
38 files sterilized 3 times without or with immersion in NaOCl. These results could be attributed to the
39 different post-machining thermo-mechanical treatment which the NiTi alloy is subjected during the
40 fabrication of Hyflex CM and TF instruments. According to the manufacturer, TF files were
41 developed by transforming a raw NiTi wire in the austenite phase into the R-phase through a
42 thermal process (10), within a very narrow temperature range (28). On the other hand, CM Wire is a
43 NiTi alloy manufactured using a special thermo-mechanical process that controls the memory of the
44 material (11,12) allowing an increased cyclic fatigue resistance (36). NiTi is a “sensitive” alloy to
45 both thermal and mechanical stresses. Patented processes are highly influenced by temperature and
46 time intervals (16). Any further machining process will affect transitional temperatures and the
47 percentage of phases of the alloy (11). This can explain why performance of NiTi rotary
48 instruments can be influenced by quality of manufacturing processes and different thermal
49 treatments.
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54 A recent study (30) reported that HyFlex CM showed an increase in cyclic fatigue resistance after
55 autoclave sterilization, while no difference was observed for TF. In addition, it was reported no
56 statistically significant difference between the two instruments in cyclic fatigue resistance. The
57 different results with our study may be explained by different instrument size, sterilization
58 conditions and methodology used (30).
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1 EDS analysis of the Hyflex CM and Twisted Files surfaces after immersion in NaOCl and
2 sterilization for 3 times revealed higher amount of oxygen and less amount of nickel on Hyflex CM
3 surface than on Twisted File's one (Fig. 2C, Sp. 3 and 2F, Sp. 3). This confirms the existence of an
4 oxide coating on shape memory files as Hyflex CM as previously reported (37). An increase of the
5 electrode potential is commonly observed in the presence of oxide layers in several alloys in
6 corrosive environments like NaOCl (37). Therefore, an increment of the corrosion resistance is
7 expected in this condition for CM-files than conventional, M-wire or R-phase files due to the oxide
8 layer that reduces the amount of nickel on instrument surface by covering it (37).
9

10 Under our testing conditions, dynamic immersion in NaOCl did not reduce significantly the cyclic
11 fatigue resistance of NiTi files examined as previously reported (21, 38). These findings may be due
12 to the type of analysis conducted. The cyclic fatigue device generates the maximum stress at the
13 center of the simulated curve (about 5 mm from the tip), so if a corrosive zone was present at that
14 level, the instrument could break early. However, if the corrosive attack hits the instrument in a
15 different area, the resistance to cyclic fatigue of the instrument will probably not be reduced (21).
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20 Within the limitations of this study, it can be concluded that repeated cycles of sterilization did not
21 influence cyclic fatigue of NiTi endodontic instruments except for the TF that demonstrated a
22 significant decrease of flexural resistance after 3 cycles of sterilization. Immersion in NaOCl did
23 not reduce significantly the cyclic fatigue resistance of both NiTi files tested.
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Legends

Table 1. Means and standard deviations (SD) of Number of Cycles to Failure (NCF) for Hyflex CM and Twisted File after immersion in sodium hypochlorite and/or autoclave sterilization.

Figure 1

FE-SEM analysis performed on fractured specimens (axial views) at 600X of Twisted Files (A-C) and Hyflex CM (D-F). Fatigue fracture surface of Twisted Files and Hyflex CM: new - not treated (A and D, respectively); dynamically immersed for 3 minutes in 5% NaOCl for 3 times (B and E, respectively); dynamically immersed in 5% NaOCl and sterilized 3 times (C and F, respectively). Similar fracture patterns (composed of distinguishable brittle and plastic areas) are visible on the same surface plane. In some samples (A, B, F) the visible parts of brittle fracture are wider (white dotted lines and arrows).

Figure 2

FE-SEM micrographs at 500X of the middle region of an HyFlex CM 25/.06 (A) and Twisted Files 25/.06 (D) dynamically immersed in 5% NaOCl and sterilized 3 times in lateral view. White rectangular area with the connected arrow represents the middle region at higher magnification (2000X) used for EDS analysis of Hyflex CM (B) and Twisted files (E). EDS spectra acquired in the rectangular marked area (Sp.3) and in 2 different points (Sp.1 and Sp.2) on HyFlex CM (E) and Twisted File (F) surface.

Table 1. Means and standard deviations (SD) of Number of Cycles to Failure (NCF) for Hyflex CM and Twisted File after immersion in sodium hypochlorite and/or autoclave sterilization.

Instrument		Hyflex CM		Twisted File	
Group	n	NCF	SD	NCF	SD
1	15	710.5 ^{a1}	71.2	705.8 ^{a1}	75.8
2	15	792.6 ^{a1}	55.8	674.4 ^{a1}	77.6
3	15	686.4 ^{a1}	63.5	661.4 ^{a1}	81.5
4	15	750.0 ^{a1}	51.5	638.9 ^{a1}	70.2
5	15	797.5 ^{a1}	65.0	484.9 ^{b2}	74.4
6	15	681.4 ^{a1}	42.6	641.9 ^{a1}	83.7
7	15	732.2 ^{a1}	74.5	548.8 ^{b2}	68.2

The same letters show differences not statistically significant ($P > .05$) in comparison with different groups of the same instrument; the same number show differences not statistically significant ($P > .05$) in comparison with the same group of different instruments

Figure
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Twisted Files 25/.06

HyFlex CM 25/.06

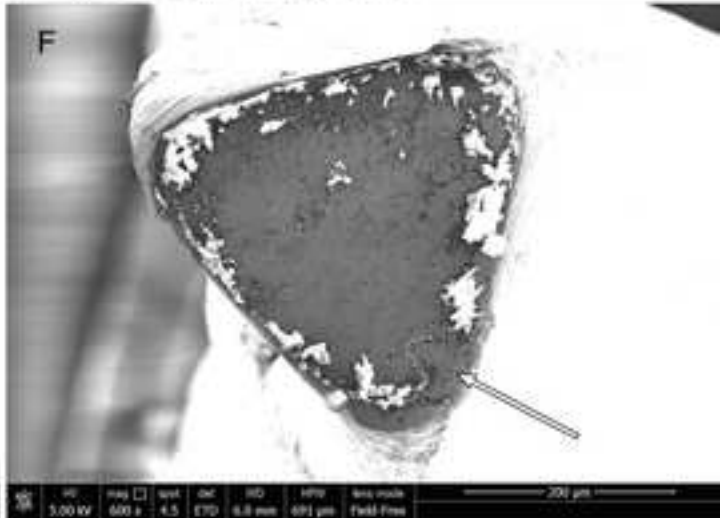
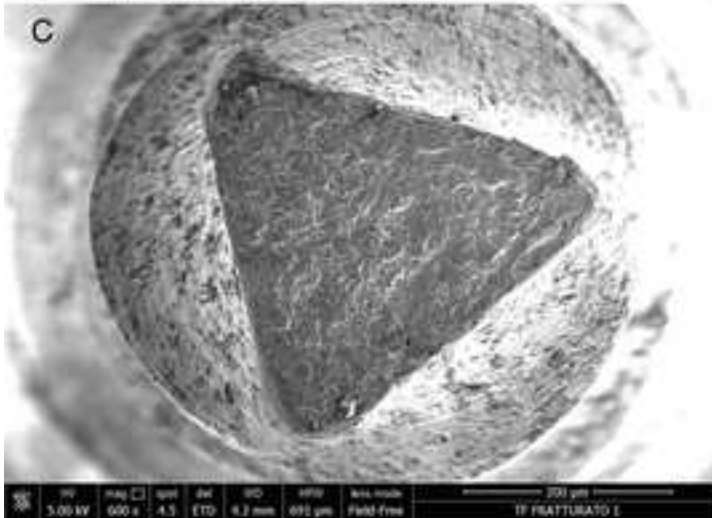
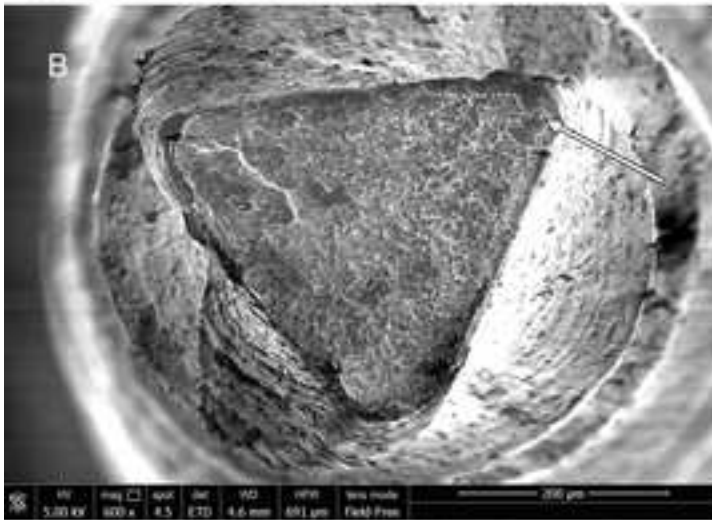
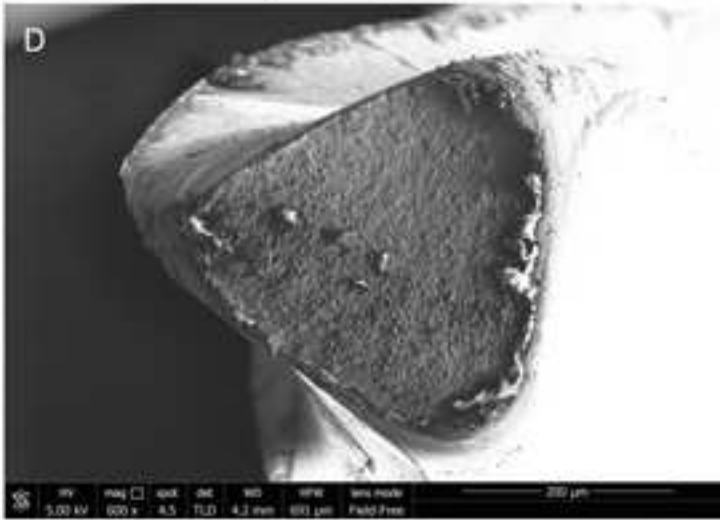
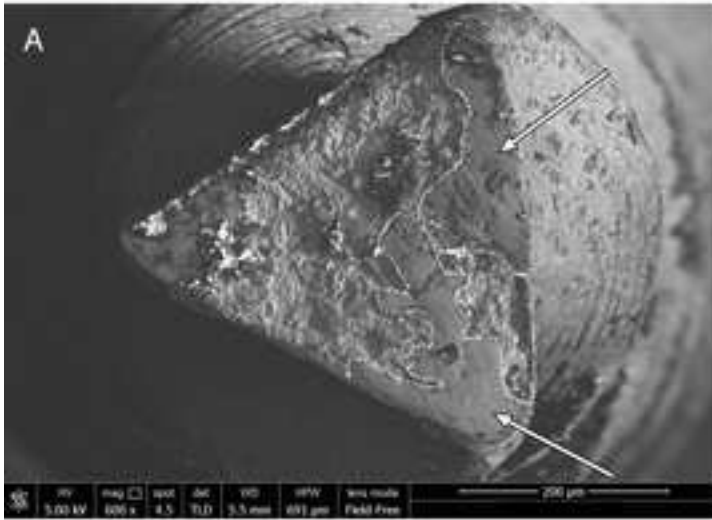


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