

A novel semi-quantitative method for measuring tissue bleeding

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Summary. In this study, we describe a new semi-quantitative method for measuring the extent of bleeding in pathohistological tissue samples. To test our novel method, we recruited 120 female patients in their first trimester of pregnancy and divided them into three groups of 40. Group I was the control group, in which no dilation was applied. Group II was an experimental group, in which dilation was performed using classical mechanical dilators. Group III was also an experimental group, in which dilation was performed using a hydraulic dilator. Tissue samples were taken from the patients' cervical canals using a Novak's probe via energetic single-step curettage prior to any dilation in Group I and after dilation in Groups II and III. After the tissue samples were prepared, light microscopy was used to obtain microphotographs at 100x magnification. The surfaces affected by bleeding were measured in the microphotographs using the *Autodesk AutoCAD 2009* program and its "polylines" function. The lines were used to mark the area around the entire sample (marked A) and to create "polyline" areas around each bleeding area on the sample (marked B). The percentage of the total area affected by bleeding was calculated using the formula: $N=Bt \times 100 / At$ where N is the percentage (%) of the tissue sample surface affected by bleeding, At (A total) is the sum of the surfaces of all of the tissue samples and Bt (B total) is the sum of all the surfaces affected by bleeding in all of the tissue samples. This

novel semi-quantitative method utilizes the *Autodesk AutoCAD 2009* program, which is simple to use and widely available, thereby offering a new, objective and precise approach to estimate the extent of bleeding in tissue samples.

Key words: Semi-quantitative method, Bleeding extent, Autodesk AutoCAD, Mechanical dilator, Hydraulic dilator

Introduction

The dilation of the cervical canal represents an invasive intervention (Bokstrom et al., 1998; O'Connell et al., 2008). Mechanical instruments, such as the Hern, Hegar, Pratt, Hanks and Denniston dilators, are used to sequentially dilate the cervix (Biron-Shental et al., 2004; Gelber and Sciscione, 2006) by incrementally increasing the diameter of the inserted dilator until the dilation procedure is complete. These mechanical dilators require the use of appropriate force, which could permanently damage the cervical tissue, thus rendering cervical dilation an invasive intervention with an associated bleeding risk (Bokstrom et al., 1998; O'Connell et al., 2008). As a replacement for mechanical dilators, we recently constructed a medical device for reliable mechanical cervical dilation: a continuous controllable balloon dilator (CCBD) with the main purpose of preventing uterine and cervical injury during cervical dilation (Arsenijevic et al., 2012).

Although recent technical advancements have been directed toward developing novel assays and methods

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for the detection and quantification of tissue injuries (Wang et al., 2011; Ippolito et al., 2012; Volarevic et al., 2012), there are still no reports of reliable, simple-to-use imaging software that could be used for the detection and quantification of hemorrhage in tissue sections.

Herein, we present a novel semi-quantitative method for measuring the extent of bleeding in cervical tissue samples obtained from patients who were dilated with mechanical or hydraulic dilators. This method was implemented using Autodesk AutoCAD 2009 software, a computer-aided design (CAD) program, which was used primarily for preparing technical drawings in 2 dimensions (2-D).

CAD in conjunction with digitized anthropometric manikins was first used in the medical field in 1990 for the analysis and control of stressful work postures (Ulin et al., 1990). Recently, AutoCAD software was successfully used to analyze human orbital asymmetry (Seiji et al., 2009) and to quantify the volume of mandibular ramus and symphysis bone grafts before surgery (Verdugo et al., 2009, 2010a), thus enhancing surgical planning prior to bone transplantation (Verdugo et al., 2010b, 2012). Additionally, this software has proven to be reliable in the semi-quantification of necrosis (Volarevic et al., 2009) and metastasis (Volarevic et al., 2012) in experimental models of hepatitis and breast cancer.

The purpose of this study was to describe a new semi-quantitative method for measuring the extent of bleeding in gynecological tissue samples. A further aim was to evaluate bleeding differences among the three groups studied: no cervical dilation, mechanical and hydraulic dilation.

Materials and methods

Study organization

We analyzed the bleeding extent in the pathohistological dilated cervical canal tissues of patients who were part of a clinical study performed from 2010 to 2011 by the Gynecology and Obstetrics Clinic of the Clinical Center of Montenegro (study no. ISRCTN54007498).

An independent data and safety monitoring board monitored the study and reviewed the protocol compliance and outcome data. The protocol was approved by the institutional review board of each participating center.

Study patients

This study was comprised of 120 pregnant women that were randomly divided into three groups. Group I (40 pregnant women) was the control group, in which no dilation was performed. Group II (40 pregnant women) was an experimental group, in which dilation was performed using a mechanical dilator. Group III (40 pregnant women) was a second experimental group, in which dilation was performed using a hydraulic dilator (Arsenijevic and Cacic, 2004; Arsenijevic et al., 2012).

The patients were enrolled in the study using the following criteria: age between 19 and 40 years; pregnancy verified by an ultrasound; singleton pregnancy; gestational age ≤ 10 months; uterus and cervix with normal findings; and absence of uterine contractions or bleeding.

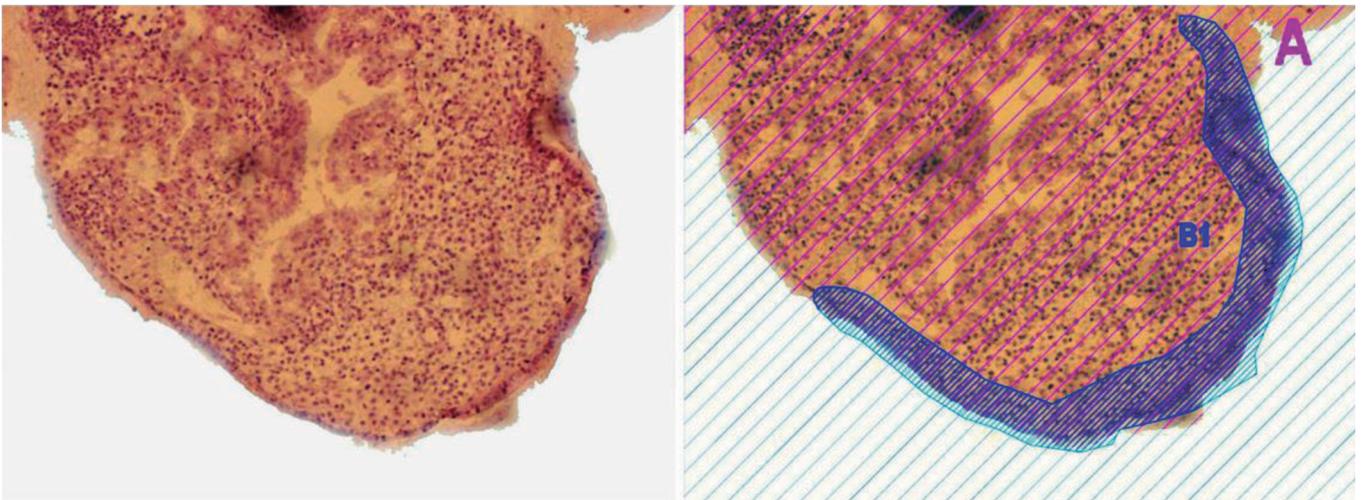


Fig. 1. The intraepithelial bleeding of the cervical canal epithelium after the application of a hydraulic dilator (left). A semi-quantitative measurement of the bleeding was calculated with Autodesk AutoCAD (right). The percentage of the tissue sample surface that was affected by bleeding was calculated as: $At=A=161.79$. $Bt=(B1)$. $Bt=(19.20)=19.20$. $N=Bt \times 100/At$. $N=19.20 \times 100/161.79$. $N=11.86\%$.

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Patients were excluded from the study if any of the following criteria were met: any signs of spontaneous abortion; any previous attempt at an abortion or use of substances for cervical maturation; multiple pregnancy; the presence or, at minimum, the suspicion of a septic abortion, followed by an elevated body temperature of 38°C or higher, uterine pain and odorous vaginal secretions; the presence of any previous intervention performed on the uterine cervix; uterine or cervical anomalies; an intrauterine device in situ; or hemorrhagic and/or chronic diseases.

Patients from each group were matched for all potentially relevant factors that could affect tissue bleeding: age, weight, smoking habits and medical history.

The experiments were undertaken with the understanding and appropriate informed consent of each patient (Arsenijevic et al., 2012).

Pathohistological analysis

The pathohistological material was taken following the preparation of the patients as if they were undergoing a routine gynecological intervention. The tissue samples were taken from the cervical canal using a Novak's probe via energetic single-step curettage. After removing the instrument, the extracted narrow belt of the epithelium was placed in a vessel with fixative solution.

To obtain relevant information and to determine the detailed structure for all three subject groups, routine hematoxylin and eosin staining was performed according to the standard protocol (Bancroft and Gamble, 2008).

The samples were fixed in a 4% buffered neutral formalin solution at room temperature for 24 hours. After the fixation was complete, the samples were dehydrated by dipping them into a graded alcohol series (70%, 96% and 100%), followed by clearing in xylol and molding in paraplast.

The samples were cut to a width of 5 μm with a Leica SM 2000R Reinhart Austria microtome. Staining

was performed using Mayer's hematoxylin followed by a 2% eosin solution. Next, the samples were dehydrated, brightened and fixed to the plates using Canada balsam. The samples were analyzed under an Olympus BX51 light microscope.

Tissue bleeding was classified as no bleeding, intraepithelial bleeding, subepithelial bleeding, stromal bleeding or combinations of the above.

Semi-quantitative method for determining the amount of bleeding

The amount of bleeding was examined by light microscopy with 100x magnification, and pictures were obtained with a digital camera. The surface area affected by bleeding was measured using the Autodesk AutoCAD 2009 software application for design and graphics (Figs. 1, 2).

The procedure that was followed using the Autodesk AutoCAD 2009 program to quantify tissue bleeding included several steps:

1. Each tissue sample area was visualized under a microscope, and the entire cervical tissue sample was photographed.

2. Each tissue sample picture was individually stored in a newly opened Autodesk AutoCAD 2009 file.

3. The "polylines" function of the program was used to draw "polyline" areas around each sample (marked as A) and around each zone on the sample that was affected by bleeding (marked as B).

4. The Autodesk AutoCAD 2009 program measured the surface of each "polyline" area. First, it measured the surface of the "polyline" A area, and then it measured the surface of each individual "polyline" B area.

5. The total area affected by bleeding was defined as a percentage using the following formula:

$$N = Bt \times 100 / At$$

where N is the percentage (%) of the tissue sample

Table 1. Frequency of patients with bleeding and classification of bleeding in the tissues.

Bleeding	Group I n(%)	Group II n(%)	Group III n(%)	Significance
No	33 (82.5%)	4 (10.0%)	27 (67.5%)	p=0.000*
Yes	7 (17.5%)	36 (90.0%)	13 (32.5%)	
Classification of bleeding in the tissues n(%)				
No bleeding	33 (82.5%)	4 (10.0%)	27 (67.5%)	p=0.000*
Intraepithelial bleeding	6 (15.0%)	4 (10.0%)	9 (22.5%)	
Subepithelial bleeding	1 (2.5%)	1 (2.5%)	0 (0%)	group I vs. II: p=0.000*
Stromal bleeding	0 (0%)	3 (7.5%)	1 (2.5%)	
Intra- + subepithelial bleeding	0 (0%)	7 (17.5%)	1 (2.5%)	group II vs. III: p=0.000*
Intraepithelial + stromal bleeding	0 (0%)	13 (32.5%)	1 (2.5%)	
Subepithelial + stromal bleeding	0 (0%)	2 (5.0%)	0 (0%)	group I vs. II: p=0.401
Intra-, subepithelial + stromal bleeding	0 (0%)	6 (15.0%)	1 (2.5%)	

*: Statistically significant.

surface affected by bleeding (Figs. 3, 4), At (A total) is the sum of the surfaces of all of the tissue samples ($At=A1 + A2 + \dots + An$, where n is the total number of photographs) and Bt (B total) is the sum of all the surfaces affected by bleeding in all of the tissue samples ($Bt=B1 + B2 + \dots + Bm$, where m is the total number of marked surfaces affected by bleeding in all of the photographs).

The percentage of the tissue surface that was affected by bleeding was calculated as an objective measurement based on the appearance of bleeding in each tissue sample, thereby avoiding the possibility of biased results due to the sample size.

Statistical analyses

The statistical analysis package SPSS 17.0 (IBM Corp., Armonk, NY, USA) was used to perform the necessary statistical tests.

Descriptive data for all of the groups and variables are expressed as the mean \pm standard deviation (SD), median, minimal and maximal values for continuous measures or percent of a group for discrete measures.

Categorical data (number of patients with bleeding and different intensities of bleeding) were analyzed using the Pearson chi-square test. A normal distribution of continuous data (area of bleeding expressed in % and total sum of bleeding surface) was analyzed using the Kolmogorov-Smirnov test. All of our data were non-parametric. The Kruskal-Wallis test was used for data analysis when more than two groups were compared. The Mann-Whitney U test was performed for inter-group comparisons.

The Spearman coefficient of correlation was calculated to show the reliability and accuracy of our novel semi-quantitative methodology with the classical pathohistology analysis.

All reported p-values were two sided. Differences

were considered significant when the p-value was <0.05 .

Results

In this study, we assessed the effectiveness of different dilators by evaluating the intensity of bleeding in cervical samples through classical pathohistological analysis and a novel semi-quantitative method. Here, we first describe our evaluation of the effectiveness of these different dilators by examining the intensity of bleeding using classical pathohistological cervical biopsy. Next, we compare our new semi-quantitative method with the pathohistological description of the bleeding intensity. Finally, we evaluate the effectiveness of the different dilators using our semi-quantitative method.

Using classical pathohistological cervical biopsy, we analyzed the impact of different dilators on the occurrence of bleeding and demonstrated that there was a statistically significant difference in the frequency of bleeding between the cervical samples from the three groups of patients (χ^2 -test, $p=0.000$) (Table 1).

The minimum frequency of bleeding was observed in the patients of Group I, and the highest frequency was observed in the patients of Group II.

Using this classical method, we then analyzed the intensity of bleeding and found a statistically significant difference between the cervical samples from the three patient groups (χ^2 -test, $p=0.000$) (Table 1).

This study describes a new method for the semi-quantitative estimation of the amount of bleeding in a tissue sample. We believe that this new method is an adequate replacement for the classical pathohistological analysis of bleeding in cervical samples. The data obtained using this classical method are descriptive and are categorized into different grade levels, as measured on an ordinal scale. Our novel method of measuring the amount of bleeding involves the use of a numerical measurement scale.

Table 2. Inter-group comparison of the surfaces affected by bleeding and percentage of bleeding area in the tissue sample.

Parameters	Group I X \pm SD; (Med; min-max)	Group II X \pm SD; (Med; min-max)	Group III X \pm SD; (Med; min-max)	Significance	
				All three groups	Between groups
Surfaces affected by bleeding (patients with bleeding)	5.63 \pm 3.42 (5.22; 1.57-10.61)	30.00 \pm 32.91 (14.70; 4.03-123)	7.80 \pm 5.77 (6.77; 1.36-24.08)	p=0.002*	^a p=0.009* ^b p=0.521 ^c p=0.006*
Surfaces affected by bleeding (all patients)	0.84 \pm 2.38 (0.0; 0.0-10.61)	27.00 \pm 32.48 (12.45; 0.0-123)	2.53 \pm 4.89 (0.0; 0.0-24.08)	p=0.000*	^a p=0.000* ^b p=0.056 ^c p=0.000*
Percentage of bleeding area in the tissue sample (patients with bleeding)	3.48 \pm 2.11 (3.22; 0.97-6.55)	12.81 \pm 11.18 (9.08; 2.5-41.67)	4.82 \pm 3.57 (4.18; 0.84-14.88)	p=0.003*	^a p=0.009* ^b p=0.521 ^c p=0.007*
Percentage of bleeding area in the tissue sample (all patients)	0.52 \pm 1.47 (0.0; 0.0-6.55)	11.53 \pm 11.28 (7.70; 0.0-41.67)	1.57 \pm 3.02 (0.0; 0.0-14.88)	p=0.000*	^a p=0.000* ^b p=0.056 ^c p=0.000*

*: Statistically significant; ^a: group I vs. II; ^b: group I vs. III; ^c: group II vs. III.

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To determine the reliability and validity of this new semi-quantitative method, we compared the data resulting from the two methods using Spearman's rank correlation coefficient (ρ), which indicate a statistically significant difference in surfaces affected by bleeding (patients with bleeding) ($\rho=0.542$, $p=0.000$) and percentage of bleeding area in the tissue sample (patients with bleeding) ($\rho=0.941$, $p=0.000$).

The advantages of the new semi-quantitative method are reliability, objectiveness and standardization of the

parameters used to evaluate the soft tissue obtained from the cervix.

Next, using our new method, we compared the bleeding area percentages in the tissue samples for the patients with bleeding in Groups I, II and III and found a statistically significant difference (Kruskal-Wallis test, $p=0.003$) (Table 2).

An inter-group comparison of the patients with bleeding confirmed that the difference in the bleeding extent was statistically significant among the tissue

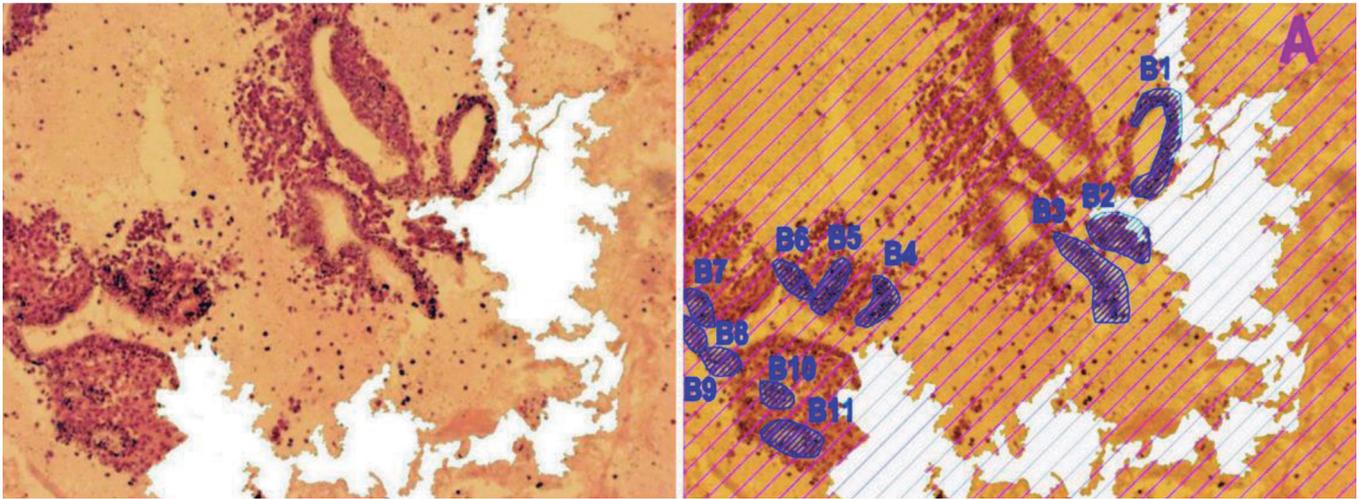


Fig. 2. The subepithelial bleeding of the cervical canal epithelium after the application of a hydraulic dilator (left). A semi-quantitative measurement of the bleeding was calculated with Autodesk AutoCAD (right). The percentage of the tissue sample surface that was affected by bleeding was calculated as: $A_t=A=161.79$, $B_t=(B_1+B_2+B_3+B_4+B_5+B_6+B_7+B_8+B_9+B_{10}+B_{11})$, $B_t=(1.30+1.05+1.45+0.55+0.55+0.42+0.38+0.30+0.4, +0.35+0.78)=7.58$, $N=B_t \times 100/A_t$, $N=7.58 \times 100/161.79$, $N=4.70\%$

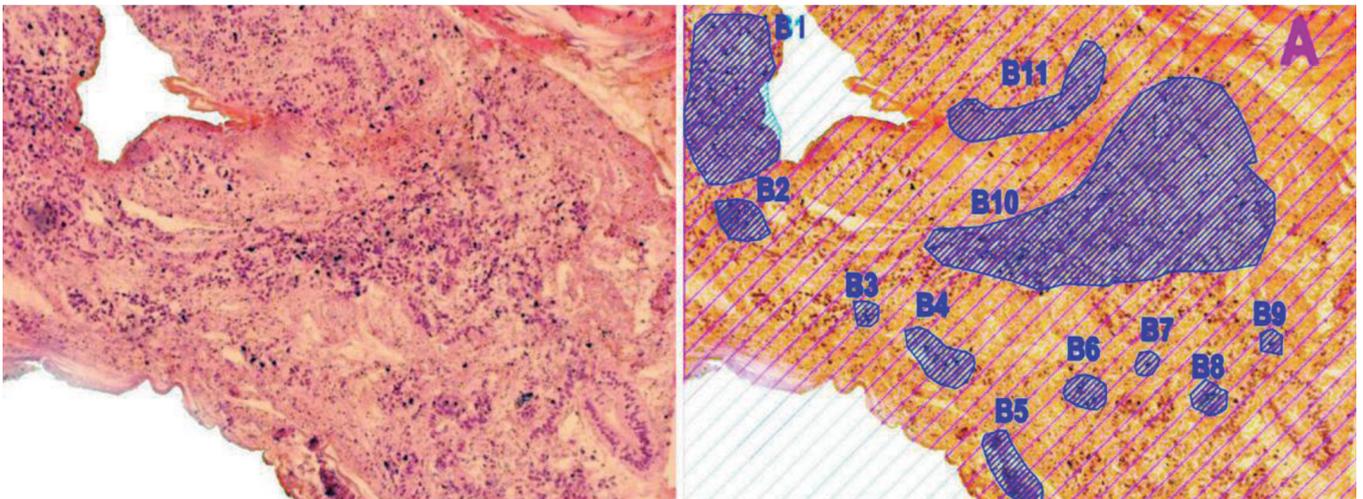


Fig. 3. The stromal bleeding of the cervical canal epithelium after the application of a mechanical dilator (left). A semi-quantitative measurement of the bleeding was calculated with Autodesk AutoCAD (right). The percentage of the tissue sample surface that was affected by bleeding was calculated as: $A_t=A=161.79$, $B_t=(B_1+B_2+B_3+B_4+B_5+B_6+B_7+B_8+B_9+B_{10}+B_{11})$, $B_t=(6.35+0.78+0.25+1.13+1.13+0.54+0.22+0.45+0.20+19.00+2.75)=32.80$, $N=B_t \times 100/A_t$, $N=32.80 \times 100/161.79$, $N=20.30\%$

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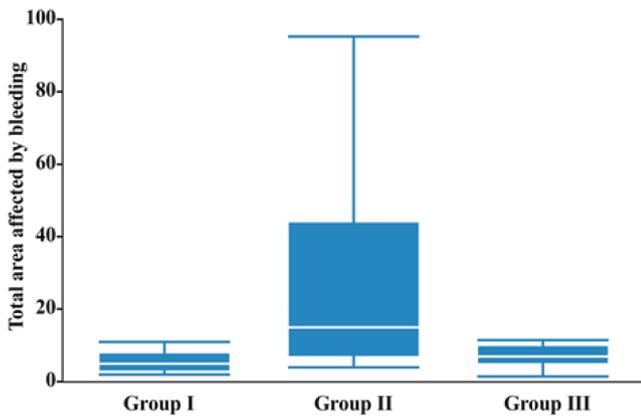
samples taken from the patients of Group I and Group II (Mann-Whitney U test; $p=0.009$). A statistically significant difference was also found between Group II and Group III (Mann-Whitney U test; $p=0.007$). However, the extent of the bleeding in the tissue samples from the patients of Group I was not significantly different from the bleeding in tissue samples from the patients in Group III (Mann-Whitney U test; $p=0.521$).

By summing the surface area of the tissue samples affected by bleeding (among the patients with bleeding) in all of the test groups, we demonstrated that the hydraulic dilator was as efficient as the mechanical dilator but caused considerably less damage to the

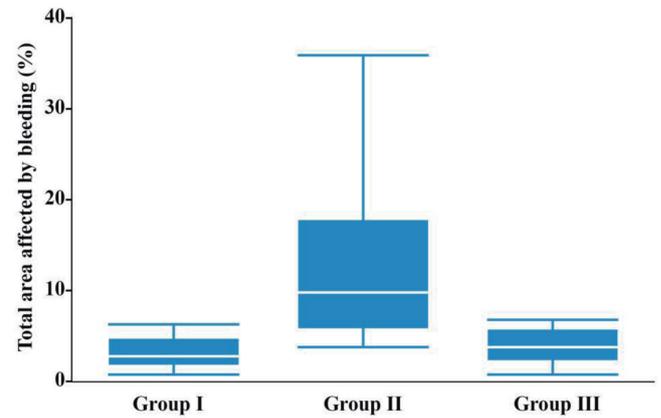
cervical tissue, as shown in Graph 1.

The statistically significant differences between the three analyzed patient groups — without dilation, after mechanical dilation and after hydraulic dilation — were also calculated with regard to the percentages of the tissue sample surface areas affected by bleeding (Kruskal-Wallis test; $p=0.003$), as shown in Graph 2.

The largest percentage of surface bleeding was found in the group of women dilated with a mechanical dilator (Group II). The percentage of bleeding in the group dilated using a hydraulic dilator was much lower (Group III); this value was similar to the percentage found for the tissues that were not dilated (Group I).



Graph 1. Total area affected by bleeding (in patients with bleeding).



Graph 2. Percentages of the total area affected by bleeding (in patients with bleeding).

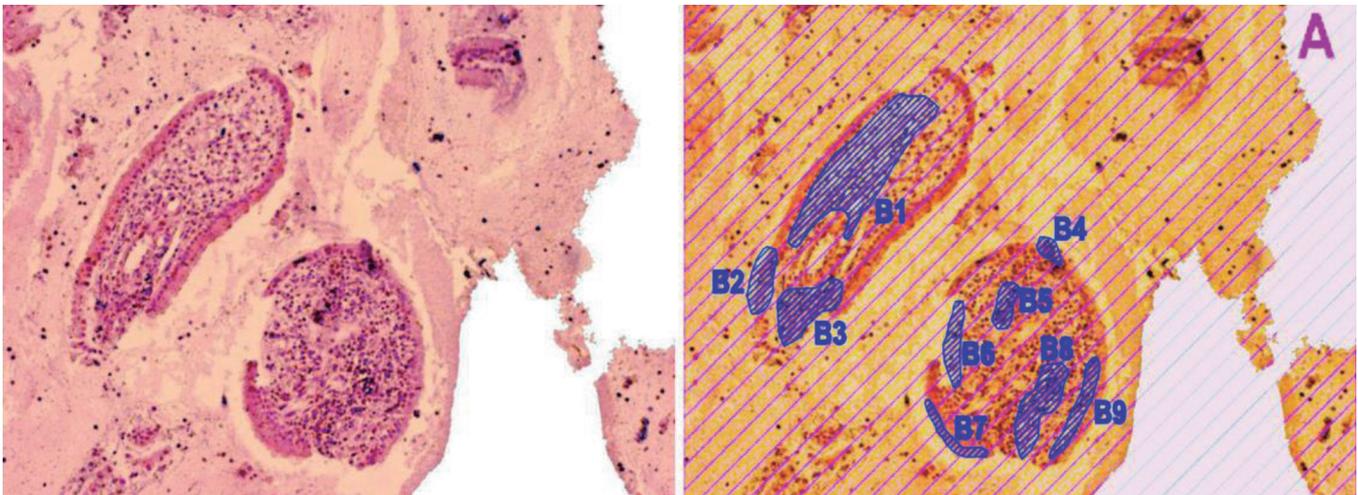


Fig. 4. The intraepithelial, subepithelial and stromal bleeding of the cervical canal epithelium after the application of a mechanical dilator (left). Semi-quantitative measurements of the bleeding were calculated with Autodesk AutoCAD (right). The percentage of the tissue sample surface that was affected by bleeding was calculated as: $A_t=A=161.79$. $B_t=(B_1+B_2+B_3+B_4+B_5+B_6+B_7+B_8+B_9)$ - $B_t=(3.79+0.63+1.13+0.19+0.37+0.53+0.33+1.12+0.58)=8.67$. $N=B_t \times 100/A_t$. $N=8.67 \times 100/161.79$. $N=5.35\%$

Discussion

Thus far, the literature has not described a method to precisely calculate the extent of tissue bleeding. Although similar methods have been used in bone transplantation (Verdugo et al., 2009, 2010a, 2012), immunology (Volarevic et al., 2009), dentistry (Unal et al., 2009), neurology (Kofron et al., 2009), anatomy (Seiji et al., 2009) and oncology (Volarevic et al., 2012), the method described in this paper represents an original model for quantifying hemorrhaging in target tissues. This model is applicable not only in gynecology (to estimate the extent of bleeding in the cervical canal) but also to estimate hemorrhages in any organ.

Additionally, histological analyses of the cervical tissue in the first (Greer et al., 1992) and third trimesters of pregnancy (Ichijo et al., 1976) have rarely been conducted.

The primary advantages of this quantitative method over other descriptive methods for hemorrhage analysis are its precision and objectivity. This method can define the extent of bleeding in an entire organ (e.g., the cervical canal, in the specific case analyzed in this paper), which is an improvement over previously described methods that only define the extent of bleeding in randomly selected tissue sample areas.

When hemorrhage is only measured in a group of representative microscopic areas (either randomly or deliberately selected), there is a high possibility for error because there might be areas affected by bleeding that are not included. Using our method, the bleeding in the entire tissue is analyzed because the Autodesk AutoCAD 2009 program automatically calculates the surface areas of the tissue affected by bleeding, thereby preventing a subjective interpretation of the results. Thus, the objectivity of the results is assured.

The reliability and objectivity of the AutoCAD software was previously proven in several studies where "polyline" areas were measured in tissue samples and radiological films.

AutoCAD was used to measure the tomographic bone volume of mandibular ramus and symphysis bone grafts (Verdugo et al., 2009, 2010a). When compared to direct surgical measurements, the AutoCAD software did not overestimate the bone volume, indicating that this program could be used to improve surgical treatment planning prior to sinus augmentation (Verdugo et al., 2010b). Additionally, AutoCAD was used to quantify necrosis in an experimental model of acute liver injury (Volarevic et al., 2009) and to estimate the percentage of lung and liver tissue parenchyma with metastases in a mouse model of breast cancer (Volarevic et al., 2012). Both studies confirmed that the data obtained with AutoCAD were precise, highly specific and similar to descriptive histological analysis, confirming the reliability and accuracy of this software for the quantification of tissue lesions.

In line with these results, we used the Autodesk AutoCAD program to demonstrate that the hydraulic

dilator does not cause significant bleeding during uterine cervix dilation; no statistically significant difference was found between the bleeding intensities of tissue samples obtained from patients with undilated uterine cervixes and those from patients who underwent hydraulic dilation. Using AutoCAD, we calculated the surface bleeding percentage and confirmed the greater efficacy of the hydraulic dilator with respect to the damage caused to the cervical tissue. Additionally, the application of this semi-quantitative method demonstrated that compared to mechanical dilators, CCBD dilation results in considerably less bleeding in the tissue of the uterine cervix.

It should also be noted that other important and significant advantages of this method are the simplicity of use of the Autodesk AutoCAD 2009 program as well as its market availability as a program that is often used for calculations and design in architecture and engineering.

Conclusion

In this study, we determined the efficiencies of different dilators with respect to tissue bleeding in cervical samples using the classical histology and novel semi-quantitative method which uses the Autodesk AutoCAD 2009 program. Both approaches have shown that the greatest level of tissue bleeding was found in patients whose dilation was performed with a mechanical dilator. Also, the amount of tissue bleeding in patients dilated with a hydraulic dilator was significantly less than patients dilated mechanically and similar to those who were not dilated.

These results suggest that the novel semi-quantitative method described in this paper which uses the Autodesk AutoCAD 2009 program, could be used as a simple and available, objective and precise approach for the estimation of bleeding in tissue samples.

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Conflict of Interest: The authors state that they have no conflicts of interest

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