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A Modified Protocol for Staining of Undecalcified Bone Samples Using Toluidine Blue—A Histological Study in Rabbit Models

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Abstract: Undecalcified bone histology is a valuable diagnostic method for studying bone microarchitecture and provides information on bone formation, resorption, and turnover. It has various clinical and research applications. Toluidine blue has been widely adopted as a staining technique for hard-tissue specimens. It provides a clear identification of bone structural and cellular features and the distinctions between them. Furthermore, the method allows for an excellent definition of the cement lines that mark the fields of bone remodeling. Some of the suggested and currently used processing and staining protocols are too complex and time-consuming, which necessitates their modification and/or optimization. This research aims to develop a simplified protocol for staining plastic-embedded undecalcified bone specimens with toluidine blue. The samples were obtained from the tibial bones of rabbits, and experiments with and without pre-etching were conducted. Our results demonstrated that the optimal visualization of the bone microstructure and its cellular components was achieved in the samples without acid pre-etching and dehydration after staining.

Keywords: bone histology; hard-tissue specimen; undecalcified bone; plastic embedding; toluidine blue staining

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1. Introduction

Histological studies of hard-tissue specimens, such as bone and teeth, are mainly performed by embedding the samples in different media—paraffin or various resin-based materials [1–4]. Traditional paraffin embedding requires the decalcification of the samples before their processing. This can be achieved using different acids (inorganic and organic) or chelating agents. Some researchers have suggested that strong inorganic acids provide rapid decalcification but could cause serious tissue damage and/or impair the staining results [5–8]. Conversely, weak organic acids and chelating agents, such as ethylenediaminetetraacetic acid (EDTA), are not that aggressive to tissues and preserve their integrity. Their utilization, however, takes considerable time; e.g., EDTA decalcification could take weeks or months, even for small sample sizes [7,8].

Recently, some combined solutions (mixtures of weak and strong acids) have been used to overcome difficulties with decalcifying hard tissues. They provide rapid decalcification (for about 6 h) without causing damage to the tissue microarchitecture [9–11].

A major limitation of paraffin embedding compared to plastic embedding is that it requires the decalcification of the specimens. Furthermore, paraffin-based media are not appropriate for embedding specimens that contain implants made of very hard materials, such as metals, ceramics, and polymers [12]. On the other hand, plastic embedding preserves the bone integrity necessary for static and dynamic histomorphometry. The method allows for the evaluation of samples that contain implants, as well as for immunohistochemical and biomechanical analyses, which are essential for some diagnostic purposes [13].

As an alternative to paraffin histology, plastic embedding has been successfully applied to the evaluation of hard tissues. It provides structural integrity and stabilization, which are necessary to perform static and dynamic histomorphometry, and also allows for immunohistochemistry [8,12–15].

The observation of both mineralized bone and unmineralized osteoid, along with their well-preserved cellular components, provides essential information about bone formation and resorption [16]. A major advantage of resin-based embedding is that it allows for the evaluation of the bone–implant interface in undecalcified sections [17–19].

Although plastic embedding does not require decalcification, it has some other disadvantages. The embedding process is multi-stage and time-consuming and involves working with dangerous and expensive reagents. In addition, the method is laborious and poses an increased risk of errors. In some cases, the complete removal of the resin is difficult, which further hinders the staining process [20–22]. Moreover, plastic embedding is incompatible with some staining techniques and can be more challenging and demanding.

Paraffin and plastic embedding have different processing and sectioning protocols. Plastic embedding does not require decalcification, and clearing is not always necessary. In addition, there are different sectioning techniques for plastic-embedded samples. The most common resin-embedding media are the following: glycol methacrylate, methyl methacrylate (MMA), and epoxy resins. When selecting an embedding medium, the hardness of the tissues must be considered. If there is a mismatch between them, this could lead to a separation between soft- and hard-tissue structures and fractures [1,2].

Plastic-based embedding medium is harder and more elastic than paraffin medium, which facilitates the sectioning of hard slices.

The sectioning of plastic-embedded samples can be performed using microtomes with special knives or via the sawing–grinding technique. The first method allows us to obtain slices with a thickness of 10 µm. When sawing and grinding, the samples are cut into thicker slices with precise saw blades and then ground and polished [23–25].

Rabbit models have been commonly used for in vivo studies of the musculoskeletal system. They are reared easily and reach skeletal maturity at a relatively early age, which limits research costs. The rabbit tibia is a preferred surgical site due to its accessibility and the small amount of surrounding soft tissues [26]. Furthermore, its size and structure correspond quite well to the edentulous human jaw, which accounts for its wide application in dental implant research [27].

There is a wide variety of staining methods for histological evaluation. Hematoxylin and eosin staining is the most common technique for the visualization of most tissue types. It is applicable to both decalcified and undecalcified sections. For the distinction between osteoid and mineralized bone, Goldner's trichrome and von Kossa staining can be used. Other common staining methods for bone histology include methylene blue fuchsin, toluidine blue, Giemsa, Masson's trichrome, etc. [28].

Toluidine blue is a cationic dye that has been widely used as a staining method for undecalcified hard-tissue sections [29–33]. It allows for the exact identification of mineralized bone matrix, osteoid, and soft tissues [32–34]. Its major advantage is the ability to identify the cement lines around osteons and hemiosteons [29–33,35]. Cement lines mark the boundaries between previously and newly formed bone, and could serve as a quantitative evaluation of the bone remodeling process. In addition, toluidine blue is successfully used for histological and histomorphometric analyses of morphological changes in the bone–implant interface [36–42].

The present research deals with the application of toluidine blue (Toluidine blue polychrome, Diapath, Italy) as a staining technique for undecalcified rabbit tibia. It aims to develop a modified protocol for staining sections with a thickness of 20–40 µm. For this purpose, experiments with and without acid pre-etching were conducted.

2. Materials and Methods

In the current research, plastic-embedded undecalcified sections from rabbit tibia were used. The embedding medium was Technovit® 9100 NEW, (Heraeus Kulzer GmbH, Wehrheim, Germany), which consists of a monomer (MMA, Basic solution), a polymer (polymethyl methacrylate—PMMA), an initiator (Hardener 1), an activator (Hardener 2), and a regulator. The procedure included several steps: fixation, dehydration, pre-infiltration, infiltration, and polymerization (Table 1).

Table 1. Laboratory protocol for processing undecalcified bone specimens.

Stages	Solution	Time [h]	Condition
Fixation	10% NBF	72	RT
Dehydration	70% EtOH	4	RT—vacuum
	80% EtOH	4	RT—vacuum
	90% EtOH	16	RT
	96% EtOH	4	RT
	96% EtOH	4	RT
	99.8% EtOH	16	RT
	99.8% EtOH	4	RT
	99.8% EtOH	4	RT
	Xylene	16	RT
	Xylene	8	RT
Pre-infiltration 1	Xylene + stabilized MMA (1:1)	24	RT—with agitation
Pre-infiltration 2	200 mL stabilized MMA + 1 g Hardener 1	24	RT—with agitation
Pre-infiltration 3	200 mL destabilized MMA + 1 g Hardener 1	24	4 °C—with agitation
Infiltration	250 mL destabilized MMA + 20 g PMMA + 1 g Hardener 1	72	4 °C—with agitation
Polymerization	Solution A + Solution B (9:1)	120	−20 °C;

RT—room temperature; EtOH—ethyl alcohol (ethanol); MMA—methyl methacrylate; PMMA—poly(methyl methacrylate).

The specimens were obtained from the tibial bones of rabbits aged 5–6 months. The soft tissues were removed, and the bone was sliced into sections with approximate length of 5 mm. Samples were fixed for 3 days in 10% buffered formalin (10% NBF, Biognost, Zagreb, Croatia). After fixation, the bones were dehydrated in a series of alcohol solutions of increasing concentration (70% EtOH, 80% EtOH, 90% EtOH, 96% EtOH, 99.8% EtOH) and cleared in xylene. Dehydration is usually performed with alcohol (ethanol) through a series of increasing concentrations that range from 70% to absolute alcohol. Otherwise, premature immersion in solutions with higher concentrations can cause tissue shrinkage and hardening. The samples were then infiltrated and embedded in the MMA-based resin Technovit® 9100 NEW (Heraeus Kulzer GmbH, Wehrheim, Germany), according to the manufacturer's instructions. The medium has a low viscosity that ensures fast infiltration. However, it demonstrates high hardness after the polymerization phase. These features make it suitable for hard-tissue histology, such as compact bone, and specimens containing medical and dental implants [1].

Preparing the solutions:

Pre-infiltration 1: Xylene and stabilized basic solution were mixed in a 1:1 ratio. The product was then transferred to a dark glass bottle and stored in a freezer at a temperature of −20 °C.

Pre-infiltration 2: One gram of Hardener 1 was added to 200 mL stabilized basic solution and mixed for 1 h using a magnetic stirrer. The product was then transferred to a dark glass bottle and stored in a freezer at a temperature of −20 °C.

Destabilization of the basic solution: For this purpose, we used column chromatography. The first step was loading the column with 50 g Al_2O_3 (Aluminium oxide 90 basic, Adsorbent for Column chromatography, Macherey-Nagel GmbH & Co.KG, Düren, Germany), and then the basic solution was passed slowly through the column. About 300 mL of MMA was destabilized in one hour. The destabilized solution could be divided into portions in dark glass bottles and stored at a temperature of −20 °C for up to 6 months.

Pre-infiltration 3: One gram of Hardener 1 was added to 200 mL destabilized MMA and mixed for 1 h using a magnetic stirrer. The product was then transferred to a dark glass bottle and stored in a freezer at a temperature of −20 °C.

Infiltration: One gram of Hardener 1 was added to 250 mL destabilized MMA and mixed for 1 h using a magnetic stirrer. Then, 20 g of PMMA (powder) was added gradually to the mixture. Each portion was added after the complete dissolution of the previous one, i.e., after a clear solution was obtained. Complete dissolution of the powder took approximately 2 h. The product was transferred to a dark glass bottle and stored in a freezer at a temperature of −20 °C.

Stock solution A: Eighty grams of PMMA were added gradually to 400 mL destabilized basic solution in a graduated glass beaker. Each portion was added after the complete dissolution of the previous one, i.e., after a clear solution was formed. Complete dissolution of the powder took approximately 6 h. Then, 3 g of Hardener 1 was added to the solution, and it was stirred for another hour. A destabilized basic solution was added to the 500 mL beaker mark and stirred for 30 min. The product was transferred to a dark glass bottle and stored in a freezer at a temperature of −20 °C.

Stock solution B: Thirty milliliters of the destabilized basic solution was added to 4 mL of Hardener 2 and stirred for 1 h in a graduated glass beaker. Then, 2 mL of the regulator was added to the solution and stirred for 30 min. The beaker was filled up to the 50 mL mark with the destabilized basic solution, and the mixture was stirred for another 30 min. The final product was transferred to a dark glass bottle and stored in a freezer at a temperature of −20 °C.

Polymerization solution: After cooling, Stock Solutions A and B were mixed in a 9:1 ratio in a glass beaker. The mixture was stirred gently for 1 min, and then it was ready for immediate use.

Polymerization:

Part of the polymerization mixture was poured into pre-cooled polyethylene embedding molds. The infiltrated tissues were then placed in the molds using plastic tweezers and covered with the polymerization solution. The loaded embedding molds were transferred to a pre-cooled desiccator (at 4 °C). Oxygen was withdrawn from the desiccator via rough vacuum (200 mBar, for 10 min) and, thus, the air bubbles formed during the pouring were evacuated. The filled molds were closed and pressed using a dental flask clamp, and placed in a freezer at a temperature of −20 °C. After 3 days, the samples were put in a refrigerator (4 °C) for 24 h. The following day, the molds were placed at room temperature for another 24 h before they were opened.

Sectioning:

Sectioning the samples at an appropriate thickness is essential for optimal light transmission. Sometimes the bone histology of specimens containing dental and orthopedic implants requires unconventional section thickness.

The hard MMA blocks containing the tissue were placed under a laboratory fume hood overnight for evaporation of the residual monomer. Then, the blocks were sectioned down to approximately 800 µm using a precision sectioning saw (Isomet 1000, Buehler Ltd., Lake Bluff, IL, USA). Sections were glued to acrylic microscope slides (Exakt, Norderstedt, Germany) using UV glue Technovit 7210 VLC (Technovit7210VLC, Heraeus Kulzer, Germany). The sections were then reduced in thickness to 20–40 µm on a grinder/polisher (EcoMet

30, Buehler, Leinfelden-Echterdingen, Germany) using Silicone Carbide abrasive papers (with grit sizes P400, P800, P1200, P1500) (CarbiMet, Buehler Ltd., Alzenau, Germany) and 0.05 µm polishing suspension (MasterPrep, Buehler Ltd., Germany).

Toluidine blue staining:

The samples thus prepared were divided into eight groups. There were a total of 120 samples—15 samples per group. The first group was stained with toluidine blue (Diapath, Italy) according to a protocol introduced by Osborne and Curtis (2005) for staining cement lines [30] (Table 2).

Table 2. The staining protocol for the identification of cement lines suggested by Osborne and Curtis [30].

Solution	Immersion Time
0.1% formic acid	5 min
dH_2O	Quick rinse
70% EtOH	15 min
Toluidine blue	5 min
dH_2O	Quick rinse
70% EtOH (differentiation)	30 s
95% EtOH	30 s
100% EtOH	30 s
100% EtOH	30 s

We conducted experiments to optimize the staining duration. The samples from groups 1, 2, 5, and 6 were etched using 0.1% formic acid for 5 min, and those from groups 1 and 5 were immersed in 70% EtOH for 15 min. The samples from groups 3, 4, 7, and 8 were initially immersed in 70% EtOH without acid pre-etching. In the next stage, the samples were stained with toluidine blue (Diapath, Martinengo, Italy). Each experimental group was divided into 5 subgroups according to their immersion time in toluidine blue—for 5, 10, 20, 30, and 40 min. Experiments with and without differentiation and dehydration were conducted. Table 3 displays the exact sequence and duration of the stages for all experimental groups.

Table 3. Experimental protocols for toluidine blue staining with and without differentiation and dehydration stages.

Experimental Group №	Immersion Time (min)						Differentiation and Dehydration
	0.1% Formic Acid	dH_2O	70% EtOH	dH_2O	Toluidine Blue (5 Subgroups According to the Immersion Time)	dH_2O	
1	5	Quickly	15	2	5; 10; 20; 30; 40	Quickly	Yes
2	5	Quickly	-	-	5; 10; 20; 30; 40	Quickly	Yes
3	-	-	15	2	5; 10; 20; 30; 40	Quickly	Yes
4	-	-	20	2	5; 10; 20; 30; 40	Quickly	Yes
5	5	Quickly	15	2	5; 10; 20; 30; 40	Quickly	No
6	5	Quickly	-	-	5; 10; 20; 30; 40	Quickly	No
7	-	-	15	2	5; 10; 20; 30; 40	Quickly	No
8	-	-	20	2	5; 10; 20; 30; 40	Quickly	No

The images were taken with a Leica MC170 HD digital camera mounted on a Leica DM1000 LED microscope (Leica Microsystems GmbH, Wetzlar, Germany). Slides were visualized using Leica Application Suite V4.13.0 software (Leica Microsystems GmbH).

3. Results

The study represents the results of eight experimental groups (Tables 4 and 5). Each experimental group was divided into five subgroups according to the immersion time in

toluidine blue. There were three samples in each subgroup and a total of 15 samples per experimental group.

Table 4. Results of toluidine blue staining (with subsequent differentiation and dehydration).

Experimental Group №	Samples	Time in Formic Acid (min)	Time in EtOH (min)	Time in Toluidine Blue (min)	Staining Result
1	1a	5	15	5	Poor
	1b	5	15	10	Poor
	1c	5	15	20	Good
	1d	5	15	30	Good
	1e	5	15	40	Good
2	2a	5	0	5	Unstained
	2b	5	0	10	Poor
	2c	5	0	20	Poor
	2d	5	0	30	Unclear
	2e	5	0	40	Unclear
3	3a	0	15	5	Unstained
	3b	0	15	10	Unstained
	3c	0	15	20	Poor
	3d	0	15	30	Poor
	3e	0	15	40	Unclear
4	4a	0	20	5	Unstained
	4b	0	20	10	Poor
	4c	0	20	20	Poor
	4d	0	20	30	Good
	4e	0	20	40	Good

Table 5. Results of toluidine blue staining (without the differentiation and dehydration stages).

Experimental Group №	Samples	Time in Formic Acid (min)	Time in EtOH (min)	Time in Toluidine Blue (min)	Staining Result
5	5a	5	15	5	Poor
	5b	5	15	10	Poor
	5c	5	15	20	Good
	5d	5	15	30	Good
	5e	5	15	40	Overstained
6	6a	5	0	5	Unstained
	6b	5	0	10	Poor
	6c	5	0	20	Poor
	6d	5	0	30	Unclear
	6e	5	0	40	Unclear
7	7a	0	15	5	Unstained
	7b	0	15	10	Unstained
	7c	0	15	20	Poor
	7d	0	15	30	Poor
	7e	0	15	40	Good
8	8a	0	20	5	Poor
	8b	0	20	10	Good
	8c	0	20	20	Good
	8d	0	20	30	Best
	8e	0	20	40	Overstained

In the first experimental group, the staining durations of 5 and 10 min led to poor staining results (pale and unclear visualization), while the samples that were immersed

in the dye for 20, 30, and 40 min demonstrated relatively good results. In the second experimental group, the staining was pale and unclear. Similar results were obtained in Group 3 and Group 4, except for the samples stained for 30 and 40 min after pre-immersion in alcohol for 20 min. Table 4 presents the results of the conducted experiments that underwent a stage of differentiation and subsequent dehydration in alcohol solutions of increasing concentration.

The staining intensity was evaluated according to the varying staining duration. Staining for 5 and 10 min resulted in pale and uneven histological images in all groups, while a longer duration (40 min) caused overstained and dark images.

Figure 1 displays photomicrographs of the experimental groups 1–4.

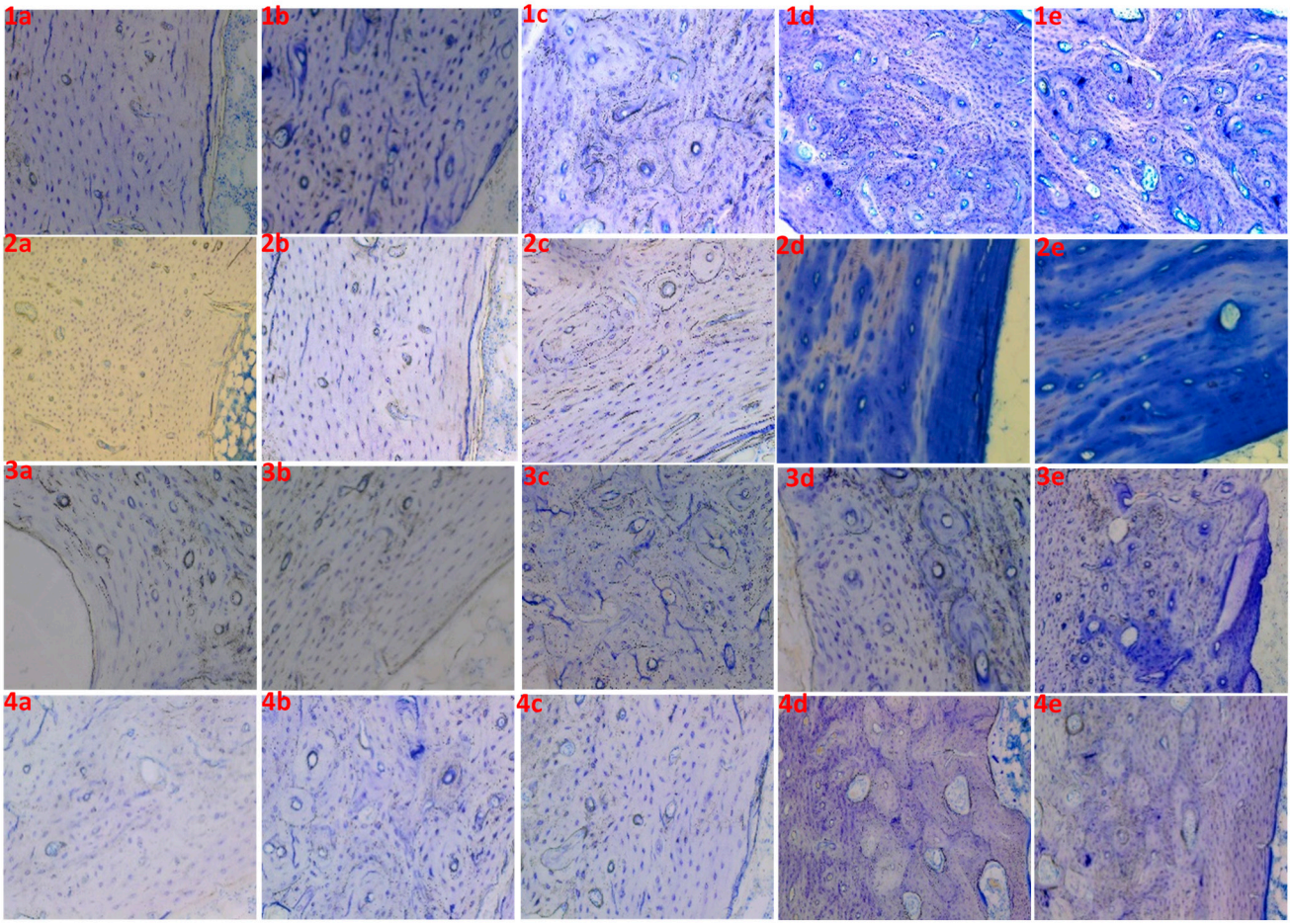


Figure 1. Photomicrographs of cross-sections through rabbit tibia (magnification 20×). The images present the results from the conducted experiments with differentiation and dehydration stages (experimental groups **1**–**4** and their subgroups **a**–**e**, respectively). The results are marked as: "poor" (**1a**,**1b**,**2b**,**2c**,**3c**,**3d**,**4b**,**4c**); "unstained" (**2a**,**3a**,**3b**,**4a**); "unclear" (**2d**,**2e**,**3e**); and "good" (**1c**–**1e**,**4d**,**4e**).

Experiments without the differentiation and dehydration stages were conducted for each group. The excess dye was removed by quickly immersing the slides in distilled water. Samples were dried for 24 h at room temperature (without pre-dehydration in alcohol), cleared in xylene, and coverslipped with BioMount DPX (Biognost, Croatia).

In Group 5, good staining results were obtained when the staining duration was 20 and 30 min, while staining for 40 min resulted in overstaining (too-dark images). In Group 6, poor and unclear staining results were obtained after etching the samples with formic acid without subsequent immersion in alcohol. The bone tissue remained unstained after staining for 5 min, while the staining durations of 10 and 20 min resulted in pale images. Increasing the staining duration in the group resulted in unclear images. For the

samples in experimental group 7, good results were obtained only after a longer staining duration—40 min. On the other hand, the samples from Group 8 demonstrated good staining results. Although immersion in the dye for 5 min led to pale staining, the results improved significantly when the staining time increased. The best results were obtained after a staining duration of 30 min. Further increasing the time led to the overstaining of the samples (Table 5). Figure 2 displays photomicrographs of the experimental groups 5–8.

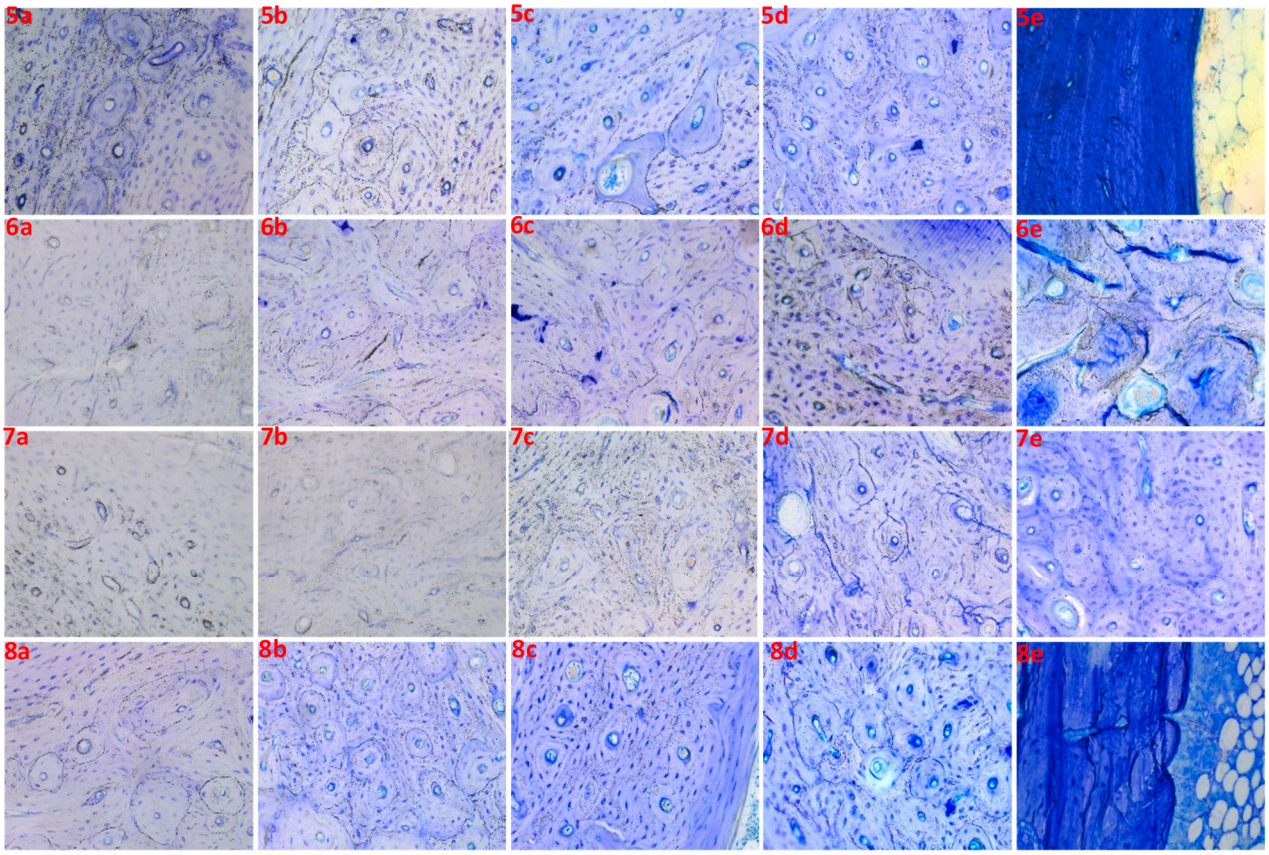
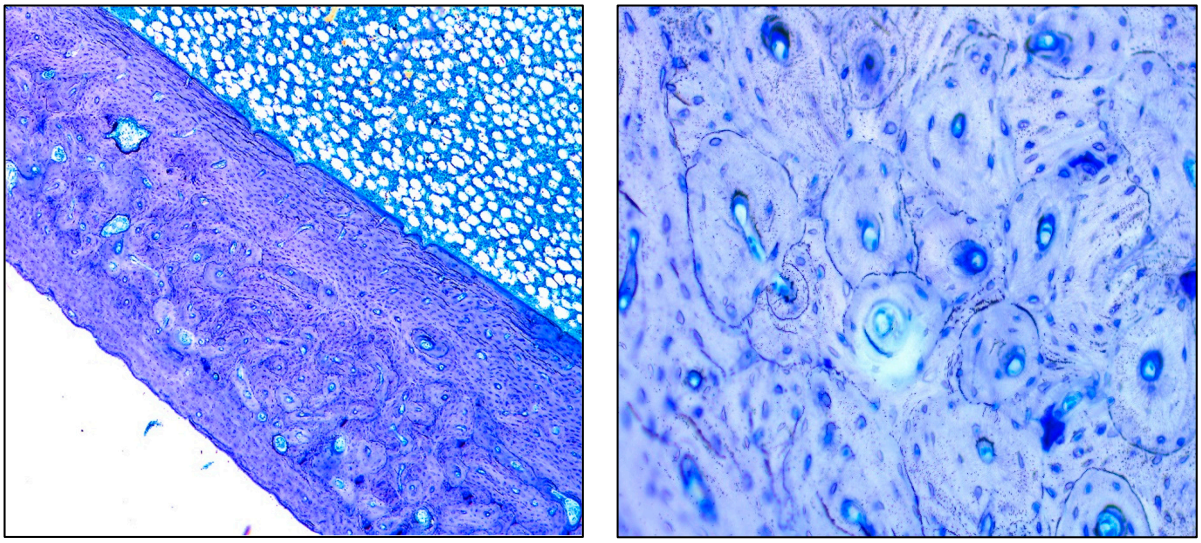


Figure 2. Photomicrographs of cross-sections through rabbit tibia (magnification 20×). The images present the results from the conducted experiments without differentiation and dehydration stages (experimental groups **5**–**8** and their subgroups **a**–**e**, respectively). The results are marked as "poor"(**5a**,**5b**,**6b**,**6c**,**7c**,**7d**,**8a**); "unstained"(**6a**,**7a**,**7b**); "overstained"(**5e**,**8e**); "unclear"(**6d**,**6e**); "good"(**5c**,**5d**,**7e**,**8b**,**8c**), and "best"—(**8d**).

In an attempt to optimize the staining protocol, we introduced a stage of washing the samples in distilled water after the immersion in ethanol, and under these conditions, more uniform staining was observed. The optimal results were obtained after pre-immersion in 70% ethyl alcohol for 20 min and staining for 30 min. The results demonstrated that good-quality images were obtained even without the differentiation stage and the subsequent dehydration in alcoholic solutions (Table 6). Usually, the role of differentiation in staining protocols is the removal of excess dye and a clear visualization of the field of interest. The suggested protocol allowed for the identification of the different cellular components, bone matrix, and cement lines that mark the fields of bone remodeling (Figure 3).

Table 6. A modified protocol for staining with toluidine blue.

Solution	Immersion Time
70% EtOH	20 min
dH_2O	2 min
Toluidine blue	30 min
dH_2O	Quick rinse
Air dry	24 h
Xylene	1 min



(**a**) (**b**)

Figure 3. Photomicrographs of cross-sections through rabbit tibia—magnification 4× (**a**) and 20× (**b**). Toluidine blue staining demonstrates osteons (Haversian systems), cement lines at their boundaries, and interstitial and circumferential lamellae.

A step-by-step graphical presentation of the optimal protocol is displayed in Figure 4.

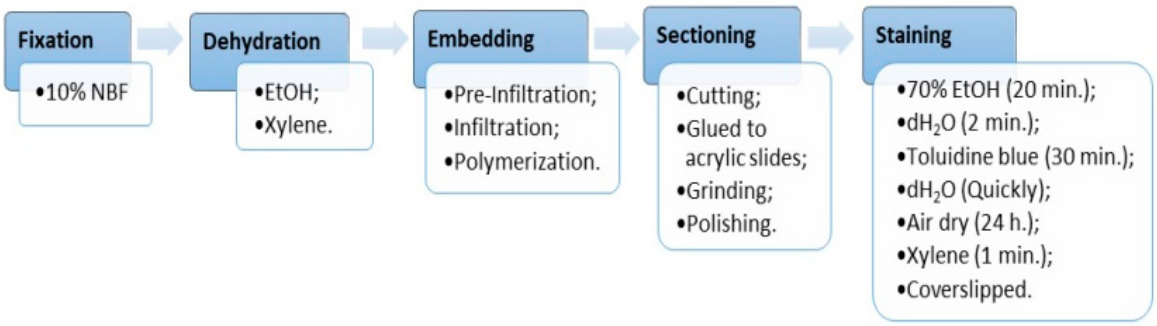


Figure 4. The stages of the suggested protocol for staining with toluidine blue.

4. Discussion

In recent years, there has been an increased utilization of orthopedic and dental implants and methods for guided bone regeneration. This has attracted the attention of re-searchers and led to a comprehensive histological evaluation of the bone–implant and bone–soft-tissue interfaces. The conventional techniques for histological observation have been insufficient for studying undecalcified bone specimens and biomaterials. This has necessitated the use of embedding media in which the integrity of both tissues and im-plants is preserved. An MMA-based medium meets these criteria. It allows a distinction

between the mineralized and unmineralized phases of bone and detailed visualization of cellular components [1].

The staining of resin-embedded samples requires the use of stains with a molecular weight that allows their passage through the resin. In this research, we used toluidine blue staining, which meets the above-mentioned requirement. It demonstrates excellent properties in the detection of cell nuclei and bone cells (osteoblast, osteocytes, and osteoclasts), as well as the non-mineralized osteoid areas.

Modifications of the staining protocols for resin-embedded sections are often necessary, since these techniques are commonly performed without the removal of the plastic. Therefore, different regimens, durations, and adjustments of the conditions could be applied [19].

The optimal staining protocol offers simplicity of execution, repeatability, cost-effectiveness, and the clear identification of different structural and cellular components and the distinctions between them.

A modified protocol for staining undecalcified bone specimens using toluidine blue is introduced in this research after qualitative evaluation of various staining techniques. The conducted experiments in rabbits aimed to validate the modified staining protocol for histological visualization of bone microstructure (osteons, cement lines, etc.) and, thus, to minimize the risk of unsatisfactory staining of human bone specimens used for research in the field of implant dentistry.

Our findings are consistent with Carter, Barnes, and Aaron [43], who also suggested a staining protocol using toluidine blue solution without acid pre-etching. However, Eurell and Sterchi [33] and Osborne and Curtis [30] reported that the acid-etching stage provides bone decalcification and is necessary for optimal staining results.

This study heterogeneity could be due to differences in the pH of the applied toluidine blue solutions. While Eurell and Sterchi [33] and Osborne and Curtis [30] used toluidine blue with pH 7.0 to 9.0, the pH of the solution used by Carter, Barnes, and Aaron [43] was 3.5 and, in our research, it was 2.0.

Moreover, it has been demonstrated that immersion in alcoholic solutions has etching properties. It softens the embedding media and allows for easier penetration of the dye [30].

These findings imply that the staining protocol can be significantly simplified by the application of toluidine blue with a lower pH.

The suggested protocol allows for the clear identification of bone matrix and cellular components using undecalcified bone slices with a thickness of 20–40 μm. Studies on implant osseointegration often use bone specimens with a thickness in this range [37,38,40].

Future research is necessary to evaluate the results after staining with toluidine blue at different concentrations and pHs, and, thus, the need for the acid pre-etching of the samples. In addition, further animal and human trials should determine the advantages and limitations of the suggested protocol in terms of simplicity, cost-effectiveness, and the quality of visualization.

5. Conclusions

Undecalcified bone histology is a valuable diagnostic tool for the evaluation of the bone microstructure. It allows for the observation and assessment of its mineral phase and cellular components, and the interfaces between bone, implants, and the surrounding soft tissues.

However, some processing and staining protocols could be technically challenging and time-consuming, necessitating their modification and optimization.

This research introduced a simplified protocol for staining undecalcified rabbit tibia using a toluidine blue solution.

It was established that the optimal results were obtained after pre-immersion of the samples in 70% ethyl alcohol for 20 min and their staining with toluidine blue for 30 min. Under these conditions, excellent visualization of bone components and microstructure was achieved.

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