ACTA VET. BRNO 2024, 93: 321–330; https://doi.org/10.2754/avb202493030321

# The effect of aldehyde fixatives, alcohol-based fixatives, and fixatives combining denaturation and crosslinking on histomorphological, histochemical, and molecular genetic studies during short-term and long-term fixation: a comprehensive analysis

Dominika Macháčová[1], Petra Konečná[1], František Tichý[1], Michal Kyllar[2], Monika Šedivcová[3], Luděk Fiala[4,5,6], Jiří Lenz[1,4,7]

[1]University of Veterinary Sciences Brno, Faculty of Veterinary Medicine, Department of Anatomy, Histology and Embryology, Brno, Czech Republic
[2]University of Veterinary Medicine Vienna, Institute of Morphology, Department of Pathobiology, Vienna, Austria
[3]Bioptical Laboratory Ltd., Pilsen, Czech Republic
[4]Cytohisto s.r.o., Břeclav, Czech Republic
[5]Charles University Pilsen, Department of Sexology, Psychiatric Clinic, Faculty of Medicine, Pilsen, Czech Republic
[6]Charles University Prague, Institute of Sexology, First Faculty of Medicine, Prague, Czech Republic
[7]Znojmo Hospital, Department of Pathology, Znojmo, Czech Republic

*Received May 3, 2024*
*Accepted July 11, 2024*

**Abstract**

The choice of fixative solution has a significant impact on the quality of tissue processing and the results of ancillary pathological studies. The present study aimed to compare the effect of different fixatives and fixative times on routinely used histopathological staining methods and molecular genetic studies in porcine endometrium. Haematoxylin and eosin staining was used to evaluate cell morphology, while Gömöri and orcein staining were selected for histochemical studies; molecular genetic studies were represented by polymerase chain reaction. Tissue samples were fixed in formalin, Bouin's fluid, methacarn, Greenfix, and Antigenfix for 1.5; 8.5; 15.5; 29.5; 64.5; 189.5; 249.5; 309.5; 369.5 days (only the period 1.5 days to 189.5 days was analyzed for histochemical studies). Formol proved to be the best for preserving cell morphology during both short-term and long-term fixation. Alcohol fixatives mainly affected the nuclear morphology, therefore they are not completely suitable for histomorphological analysis. The best preservation of nuclear morphology was achieved with Bouin's fixative. For histochemical studies and short-term fixation, fixative solutions of all 3 groups (crosslinking, denaturing fixatives and fixatives combining both denaturing and cross-linking effects) were suitable. For long-term fixation, the alcohol fixative methacarn proved to be the best in histochemical studies. Finally, for molecular genetic studies, methacarn was the best fixative, while Bouin's fluid was completely unsuitable. Formalin was confirmed as the most universal fixative overall. However, the selection of a suitable fixative should always be made depending on the specific pathological study and the fixation time.

*Cell morphology, ancillary studies, Antigenfix, Greenfix*

Tissue fixation is a very important step in the handling of biopsy material, allowing further processing of the tissues (Fox et al. 1985). Important requirements for fixative solutions include preservation of tissue antigenicity and applicability for different tissue types as well as for subsequent ancillary studies.

Based on the mechanisms of fixation, fixative solutions can be divided into four groups (Bancroft and Stevens 1996). The first group includes crosslinking fixatives, which act through addition and formation of intermolecular and intramolecular crosslinking bonds. This type of fixatives affects the molecular conformation, solubility, and staining characteristics of proteins. For example, formaldehyde, the most widely used crosslinking fixative, reacts extensively with amino groups to form methylene bridges, thereby reducing the availability of these groups to bind negatively charged molecules such as

**Address for correspondence:**
Jiří Lenz
Department of Pathology, Znojmo Hospital
MUDr. Jana Janského 11, Znojmo,
669 02, Czech Republic

Phone: +420 515 215 478
E-mail: jiri.lenz@gmail.com
http://actavet.vfu.cz/

eosin (Srinivasan et al. 2002). The second group of fixative solutions are denaturing (or precipitating) fixatives (e.g., Carnoy's or methacarn), which destabilize hydrophilic and hydrophobic bonds, thereby causing changes in the tertiary structure of proteins (Eltoum et al. 2001). Oxidizing fixatives, such as osmium oxide or potassium permanganate, belong to the third group of fixative solutions. They cause both the formation of crosslinks and the denaturation of proteins. The fourth and final group of fixative solutions are metallic fixatives, which act through the formation of an insoluble metal precipitate.

Not only the type of fixative but also the fixation time significantly affect the results of routinely used histopathological staining methods. Underfixation leads to autolytic and putrefactive processes in deeper tissue structures, as well as artifacts arising from suboptimal processing and uneven tissue staining (Bindhu et al. 2013). On the other hand, overfixation causes hardening and shrinkage of the tissue samples, structural alterations and damage of antigenic sites in tissues.

The aim of this study was to investigate the effect of different types of fixative solutions and fixation time on cell morphology and selected histochemical and molecular-genetic studies in porcine endometrial tissue. Haematoxylin-eosin (H&E) staining was used to evaluate cell morphology, while Gömöri trichrome and orcein staining were selected for the histochemical studies. Polymerase chain reaction (PCR) analyzing the *SOX2* gene was chosen from the group of molecular genetic studies.

## Materials and Methods

### Processing, fixation, storage and submitting of tissues

Our study comprised a total of 10 tissue samples taken from hysterectomy specimens collected from healthy sows in an animal abattoir. Immediately after collection, the tissue specimens were transported and processed at the Department of Histology, Embryology and Anatomy of the University of Veterinary Sciences Brno. Tissue fragments approximately 10 × 10 × 15 mm in size were dissected from the middle part of each of the uterine horns. Subsequently, each of the fragments was divided into 5 equal parts and each part was placed in a container filled with one of the fixatives to be assessed, namely, 10% neutral buffered formalin (NBF), Antigenfix (both crosslinking fixatives), methacarn (denaturing fixative), Bouin's solution and Greenfix (both combining denaturing and cross-linking effects). Specification of the fixative solutions employed was reported previously (Lenz et al. 2022a). The volume of the fixative solution was 10 to 15 times that of the tissue being fixed. Samples were designated and stored in a permanent storage area at room temperature of 20–23 °C. For H&E staining as well as for the molecular genetic study, the submitting of tissue fragments was performed over a total of 9 fixative periods, specifically after 1.5 days; 8.5 days (1 week); 15.5 days (2 weeks); 29.5 days (1 month); 64.5 days (2 months); 189.5 days (6 months); 249.5 days (8 months); 309.5 days (10 months) and 369.5 days (12 months/ 1 year) days of fixation. For Gömöri and orcein staining, 6 fixative periods from 1.5 to 189.5 days of fixation were analyzed. Before analysis, H&E-stained slides were reviewed by a pathologist (JL) to rule out endometrial and/or myometrial pathology.

### Haematoxylin and eosin staining

Slides were stained with H&E as described previously (Lenz et al. 2022b).

### Gömöri staining

Slides were dewaxed and rehydrated, oxidised with 1% potassium permanganate (2 min), treated with 3% potassium pyrosulphite and 3% iron alum solution (1 min), washed with water and distilled water (5 min each), treated with ammoniacal silver (1 min) and reduced formol, toned with 1% gold chloride solution (5 min) and fixed with 5% sodium thiosulphate (1 min), washed with water and dehydrated in 2 changes of ethanol and xylene. Finally, the slides were coverslipped with mounting medium solacryl. Gömöri silver stain labels reticulin fibres black.

### Orcein staining

The slides were dewaxed and rehydrated in 2 changes of xylene and ethanol (5 min each), washed with water, stained with orcein (6 h at a temperature of 38 °C), washed with distilled water and differentiated with acid ethanol (70%), stained with haematoxylin (5 min), washed with water and dehydrated in 2 changes of ethanol and xylene. The slides were then coverslipped with mounting medium solacryl. Orcein stain labels elastic fibres brick red.

### Molecular genetics

DNA from fixed tissues was extracted using QIAsymphony DNA Mini Kit (Qiagen, Hilden, Germany) on an automated extraction system (QIAsymphony SP, Qiagen) according to the manufacturer's supplementary

protocol for FFPE samples (Purification of genomic DNA from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution). Concentration and purity of isolated DNA was measured using NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.). DNA integrity was examined by amplification of control gene *SOX2* in a multiplex PCR.

The *SOX2* gene amplification was performed using PCR. Briefly, 100 ng DNA was added to a reaction mixture consisting of 10 µl of FastStart™ PCR Master (Sigma-Aldrich, Missoury, USA), 10 pmol of forward and reverse primers (Table 1), $Mg^{2+}$ (25 mM) and distilled water up to 20 µl. The amplification program comprised denaturation at 95 °C for 9 min, 40 cycles of denaturation at 95 °C for 1 min, annealing 60 °C for 1 min and extension at 72 °C for 1 min. The program was terminated by incubation at 72 °C for 7 min. The PCR products were separated by electrophoresis on a 2% agarose gel.

Table 1. Summary of *SOX2* gene primers used to examine DNA integrity in multiplex PCR.

| PCR primer | Primer sequences | Product size (bp) |
|---|---|---|
| SOX2-F 100 bp | GCAAGATGGCCCAAGAGAA | 116 |
| SOX2-R 100 bp | CTTGGCCTCGTCGATGAAC | |
| SOX2-F 300 bp | AAGACGCTCATGAAGAAGGA | 306 |
| SOX2-R 300 bp | GTTCATGTAGGTCTGCGAGC | |
| SOX2-F 500 bp | CGGAAAACCAAGACGCTCAT | 498 |
| SOX2-R 500 bp | GGGGAGGTACATGCTGATCA | |

bp - base pair

### Evaluation of haematoxylin and eosin staining

H&E staining was used to assess cell morphology. The following indicators were evaluated: 1) distinction between individual cell types; 2) recognition of the cell nucleus; 3) recognition of nuclear details (specifically, the shape of nuclei, chromatin structure, presence of nucleoli and mitotic figures); 4) cytoplasmic retraction; 5) pericellular retraction; and 6) overall staining (merging of all these indicators including colour changes). Except for the nuclear details, a three-tiered scoring system (0 through 2) was employed with the corresponding terminology: 0 – unsatisfactory (barely discernible at low magnification); 1 – satisfactory (not easily discernible at low magnification); 2 – good (easily discernible at low magnification) (for indicators 1, 2, 3 and 6) and 0 – absent; 1 – mild (not easily discernible at low magnification); 2 – considerable (easily discernible at low magnification) (for indicators 4 and 5). For nuclear details only, one more assessment was added: 3 – very good (very easily discernible at low magnification).

### Evaluation of histochemical staining

For both Gömöri and orcein staining, the following indicators were evaluated using a four-tiered scoring system: 1) staining intensity, 2) contrast, and 3) sharpness; scale 0 – unsatisfactory, 1 – satisfactory, 2 – good or 3 – very good.

### Statistical analysis

For individual fixatives, the differences of the analyzed indicators at various time intervals were compared using the Wilcoxon signed rank test with continuity correction. $P = 0.05$ was used as the level of significance in all analyses.

## Results

### Morphology

#### Neutral buffered formalin

In formalin-fixed tissues, a good distinction between individual cell types and recognition of the cell nucleus was preserved in all monitored periods (mean scores 2 and 1.8, respectively). Recognition of nuclear details was evaluated as good across fixation intervals (mean score 1.9). Considerable cytoplasmic retraction (mean score 2.0) and mild pericellular retraction (mean score 1.3) were observed during the study period. Overall staining was good regardless of fixation time (mean score 1.8) (Plate VIII, Fig. 1A,B).

#### Antigenfix

When using Antigenfix, the distinction of individual cell types was evaluated as good after 1.5 and 15.5 days of fixation (mean score 2.0) and as satisfactory in the remaining

monitored periods (mean score 1.2). During all fixation times, recognition of the cell nucleus was only satisfactory (mean score 1.1). Regarding the nuclear details, these were assessed as satisfactory throughout the period of the study (mean score 1.5). Regardless of the fixation interval, mild pericellular and perinuclear retraction was observed (mean scores 0.9) and overall staining was evaluated as satisfactory (mean score 1.0) (Plate VIII, Fig. 1C,D).

Methacarn

In methacarn-fixed tissues, the distinction of individual cell types was good between 1.5 and 64.5 days (mean score 1.9) and satisfactory between 189.5 and 369.5 days of fixation (mean score 1.1). Both recognition of the cell nucleus and assessment of nuclear details were satisfactory across all fixation periods (mean scores 0.9 and 1.0, respectively). Cytoplasmic as well as pericellular retraction were practically not present in any of the monitored periods (mean score 0.2 and 0.0, respectively). Between 1.5 and 64.5 days, overall staining was evaluated as satisfactory (mean score 1.1) and then between 189.5–369.5 days of fixation as unsatisfactory (mean score 0.2) (Plate VIII, Fig. 1E,F).

Bouin

Bouin's fluid allowed good distinction between individual cell types across fixation times as well as distinction of the cell nucleus (mean scores 2.0 and 1.9, respectively). Nuclear details were assessed as very good between 1.5 and 309.5 days (mean score 2.9) and as good after 369.5 days of fixation (mean score 2.2); differences were noted especially in leiomyocytes. During the study period, cytoplasmic retraction was virtually absent (mean score 0.1) and pericellular retraction was mild (mean score 1.1). In contrast to the 1.5 days of fixation when the overall staining was good (total score 2.0), the remaining fixation times were assessed as only satisfactory due to fading (mean score 1.0). Moreover, as the fixation time increased, the staining became more pink and red (Plate VIII, Fig. 1G,H).

Greenfix

When using Greenfix, a good distinction of individual cell types was possible during the entire monitored period (average score 2.0). A slight decrease occurred after 189 and 369 days of fixation (mean score 2.2 between 1.5 and 64.5 days, 2.0 between 189 and 309 days, and 1.8 after 369 days of fixation). Cell nucleus recognition was evaluated as satisfactory (mean score 1.0) with a slight decrease after 309 days of fixation (mean score 0.7). Assessment of nuclear details ranged between good and satisfactory (mean score 1.5; score range 1.6–1.4), with the greatest differences being in glandular cells. While cytoplasmic retraction was hardly observed (mean score 0.1), pericellular retraction was considerable during the study period (mean score 1.9). Overall staining had a mean score of 0.8 corresponding to satisfactory (Plate IX, Fig. 1I,J).

## Histochemistry

### Gömmöri

Formol

Staining intensity, epithelial-stromal contrast, and sharpness showed a significant decrease over time. All three analyzed indicators were evaluated as very good after 1.5 days of fixation (mean scores 2.9, 2.8 and 2.9, respectively). Staining intensity and sharpness then decreased slightly to good during 8.5 and 15.5 days of fixation (mean scores 2.0 and 2.1, respectively). After 29.5 days, sharpness was still assessed as good (mean score 1.8), while intensity significantly decreased to satisfactory (mean score 1.0; $P < 0.001$). After 64.5 days of fixation, sharpness also decreased significantly (mean score 1.0; $P < 0.001$). Both of these indicators were evaluated as unsatisfactory after 189.5 days

of fixation (mean scores for both indicators 0.2; $P < 0.001$). Epithelial-stromal contrast decreased significantly after 8.5 days (mean score 1.3) and was evaluated as unsatisfactory after 189.5 days of fixation (mean score 0.2; $P < 0.001$ for both mentioned fixation times) (Plate IX, Fig. 2A,B).

Antigenfix

All analyzed indicators were evaluated as very good after 1.5 and 64.5 days (mean scores 2.9, 2.8 and 2.9, respectively). After 189.5 days of fixation, intensity and epithelial-stromal contrast slightly decreased to good (mean scores 2.1 and 2.0, respectively), while sharpness remained virtually unchanged (mean score 2.8) (Plate IX, Fig. 2C,D).

Methacarn

In methacarn-fixed tissues, staining intensity was assessed as good (mean score 2.0, score range 2.2–1.8), epithelial-stromal contrast as very good (mean score 2.8, score range 3.0–2.7), and sharpness as satisfactory (mean score 1.0, score range 1.2–0.8) throughout the study. No significant effect of fixation time was noted for any of the observed indicators (Plate IX, Fig. 2E,F).

Bouin

Using Bouin's fluid, all indicators were evaluated as very good after 1.5 and 15.5 days (mean scores 2.9, 2.9, and 3.0, respectively) and as good after 29.5 and 64.5 days of fixation (mean scores 2.2, 2.2, and 2.1, respectively). After 189.5 days of fixation, a significant decrease was noted in epithelial-stromal contrast (mean score 0.9; $P < 0.001$), whereas intensity and sharpness decreased only slightly (mean scores 1.8 and 2.0, respectively). With Bouin's fixative, the effect of long-term fixation on the result of histochemical/Gömöri staining was demonstrated (Plate IX, Fig. 2G,H).

Greenfix

For tissues fixed with Greenfix, all three indicators were evaluated as very good over the entire monitored period (mean scores 2.9 for all indicators, score range 3.0–2.7). No effect of fixation time on intensity, contrast or sharpness was observed (Plate IX, Fig. 2I, J).

Orcein

Formol

Evaluation of orcein staining in formalin-fixed tissues showed that staining intensity and epithelial-stromal contrast were assessed as good after 1.5 days (mean scores 2.1 and 2.2, respectively), satisfactory in the period of 8.5 to 29.5 days (mean scores 0.9 and 1.0, respectively), and unsatisfactory in the remaining time periods (mean scores 0.3); the differences reached significance ($P < 0.001$). Sharpness was very good after 1.5 days of fixation (mean score 3.0), in the period 8.5 to 29.5 days it subsequently decreased to only good (mean score 1.9) and after 64.5 days it was evaluated as unsatisfactory (mean score 0.2; $P < 0.001$). The effect of fixation time on histochemical/orcein staining was demonstrated (Plate X, Fig. 3A,B).

Antigenfix

In Antigenfix-fixed tissues, intensity and sharpness remained unchanged throughout the study, with intensity assessed as good (mean score 2.2) and sharpness as very good (mean score 2.9). In epithelial-stromal contrast, after 189.5 days of fixation there was a decrease from the assessment of good (mean score 2.2) to satisfactory (mean score 1.3). Thus, the effect of long-term fixation with Antigenfix was demonstrated only on the contrast (Plate X, Fig. 3C,D).

Methacarn

When using methacarn fixative, fixation time had no effect on any of the analyzed indicators. While sharpness was very good (mean score 2.9), intensity and epithelial-stromal contrast were assessed as good (mean scores 1.9 and 2.0, respectively) (Plate X, Fig. 3E,F).

Bouin

All analyzed indicators were evaluated as very good in the period 1.5 to 29.5 days (mean scores 3.0, 2.9 and 2.9, respectively), as only good after 64.5 days (mean scores for all indicators 2.2), with a significant decrease observed after 189.5 days of fixation (mean scores for all indicators 0.8; $P < 0.001$). Using Bouin's fluid, the effect of long-term fixation on histochemical/orcein staining was demonstrated (Plate X, Fig. 3G,H).

Greenfix

In Greenfix-fixed tissues, staining intensity was assessed as good throughout the study period (mean score 2.1). Epithelial-stromal contrast and sharpness were evaluated as very good after 8.5 days (mean scores for both indicators 2.8) and as good during the remaining fixation intervals (mean scores for both indicators 2.2) (Plate X, Fig. 3I,J).

## Molecular genetics

Formol

In formalin-fixed tissues, DNA concentration ranged from 28.91 to 3.99 ng/µl. A decrease was noted after 249.5 days (14.65 ng/µl), and after 369.5 of fixation, the decrease reached statistical significance (3.99 ng/µl; $P < 0.001$). DNA purity measured by an absorbance ratio of 260/280 nm (A260/280) was within the normal range (1.76–1.73) during the period of 1.5 to 249.5 days of fixation. A secondary indication of sample purity, which was assessed by an absorbance ratio of 260/230 nm (A260/230), was outside normal values during the study. Regarding PCR amplification, the 116 bp fragment could be amplified during all time intervals, while the 306 bp fragment was only amplified during 1.5 to 64.5 days of fixation and the 498 bp fragment could not be amplified at all.

Antigenfix

When using Antigenfix, the highest DNA concentration (53.43 ng/µl) was reached after 1.5 days of fixation. A significant decrease to 30.42 ng/µl occurred after only 8.5 days ($P < 0.001$). In the period of 15.5 to 369.5 days of fixation, there was a gradual decrease in the concentration in the range of 28.83 to 12.37 ng/µl. Based on A260/280, DNA of good purity could only be obtained in the period between 1.5 and 15.5 days of fixation (1.73–1.75). In the remaining time periods, it was no longer possible to isolate DNA of good purity. A260/230 was outside the normal range throughout the study. Amplification of the 116 bp fragment was possible during all fixative periods. A 306 bp fragment could be amplified during the entire monitored period except for 369.5 days. Finally, a 498 bp fragment could only be amplified at 1.5 days of fixation.

Greenfix

After fixation with Greenfix, the DNA concentration during the observed period ranged from 79.09 to 11.90 ng/µl. A significant decrease occurred after 29.5 days of fixation from the original 79.09 ng/µl to 25.94 ng/µl. Later in the monitored period, the DNA concentration gradually decreased to a value of 11.90 ng/µl after 369.5 days of fixation. While A260/280 was within the normal range during 1.5 to 15.5 days of fixation, A260/230 was outside the normal range throughout the whole period (1.84–0.95). In tissues fixed by Greenfix, all three fragments were amplified at 1.5 days of fixation. Furthermore, the 116 bp fragment was amplified within 15.5 days of fixation, after which amplification was already negative.

Bouin

The concentration of DNA after fixation in Bouin's solution reached only 3.32 ng/µl at 1.5 days of fixation, then gradually decreased to −0.23 ng/µl after 369.5 days of fixation. The absorbance ratios (A260/280 and A260/230) were outside the normal range in all monitored periods. Amplification could not be performed at any of the fixation times (this applies to all three fragments).

Methacarn

When using methacarn, the concentration of isolated DNA in the period of 1.5 to 29.5 days was in the range of 112.04–95.86 ng/µl. The first significant decrease occurred after 64.5 days (79.90 ng/µl; $P < 0.001$), then also after 249.5 days (52.36 ng/µl; $P < 0.001$) and 309.5 days (29.16 ng/µl; $P < 0.001$). After 369.5 days of fixation, the DNA concentration was 31.54 ng/µl. According to A260/280, DNA of good purity was obtained throughout the monitored period. A230/260 reached normal values only at 1.5 days of fixation. In methacarn-fixed tissues, the 116 bp and 306 bp fragments were amplifiable during all time intervals, with the 498 bp fragment amplified in the period up to 249.5 days of fixation.

Tables that support the findings of this study are available from the corresponding author upon reasonable request.

## Discussion

Although the assessment of cell/tissue morphology is still considered the gold standard for histopathological diagnosis, molecular biological studies are still on the rise in recent years. Most diagnostically important pathological studies are performed on routinely processed formalin-fixed paraffin-embedded (FFPE) tissues. However, formalin is generally known to have negative effects on nucleic acids, and therefore the search for alternative fixatives is an active area of scientific investigation (Netto et al. 2003).

According to the results of our current study, formalin is the most suitable fixative solution for histomorphological analysis. Another suitable fixative is Bouin's fluid, where nuclear details were even better preserved compared to formalin, but overall staining was poorer. Third in the order was Greenfix, which was worse than Bouin's solution, especially in the assessment of nuclear details. In addition, considerable pericellular retraction in Greenfix-fixed tissues closely mimics lymphovascular invasion. Very similar results as with Greenfix were achieved with Antigenfix. Methacarn was evaluated as the worst fixative of all for histomorphological analysis; its use affected nuclear details the most. However, the advantage of methacarn tissue fixation is the absence of cytoplasmic and pericelullar retraction.

The finding that formalin is the most suitable fixative for evaluating cell/tissue morphology was also reported in a study by Matsuda et al. (2011), who compared formalin, paraformaldehyde, and 99% ethanol fixatives. The authors concluded that 99% ethanol is a significantly inferior fixative solution for histomorphological analysis compared to formalin, particularly in terms of preservation of nuclear details, appearance of cytoplasmic eosinophilia and cell contraction. These results are consistent with our current study, however, we did not observe pericellular retraction when using methacarn fixative.

In contrast to our current study and that performed by Matsuda et al. (2011), several studies highlight the benefits of alcohol fixatives for preserving cell morphology. For example, Meecham et al. (2021) reported comparable results of histomorphological analysis during a short-term 24-h formalin fixation and PAXgene alcohol fixation, as well as similar values of DNA concentrations extracted from tissue samples.

Apart from our current study, only Gatta et al. (2012) dealt with the effect of Greenfix fixation on pathological studies with somewhat conflicting results. This

fixative combines both coagulation and crosslinking effects. In contrast to our study, the authors demonstrated a similar effect of short-term 24-h fixation when using formalin and Greenfix on histomorphological analysis. We found worse overall staining in tissues fixed with Greenfix, compared to formalin, the recognition of the nucleus and nuclear details was also worse. The only advantage of Greenfix was the absence of cytoplasmic retraction, which was considerable during both short-term and long-term formalin fixation. To our knowledge, the present study is the first to address the effect of fixation time on ancillary studies using the Greenfix fixative. We have demonstrated that the fixation time is not a factor significantly affecting the morphological features of tissues fixed in Greenfix. Regarding molecular genetic studies, both our study and that of Gatta et al. (2012) showed that Greenfix is more suitable than formalin for short-term fixation. However, formalin was a surprisingly better fixative for long-term fixation in our study. Also for amplification, formalin was superior to Greenfix in terms of long-term fixation. Finally, both our study and that of Gatta et al. (2012) consider Greenfix to be comparable or slightly superior to formalin for histochemical studies.

To the best of our knowledge, the present study is the first ever to describe the effect of the crosslinking fixative Antigenfix on pathological studies (with the exception of immunohistochemical studies that we analyzed in our recent study [Lenz et al. 2022a]). This fixative appeared to be significantly inferior compared to formalin for evaluation of cell morphology, but as with formalin, it was noted that fixation time had no effect. The negative effect of Antigenfix on histomorphological analysis can be attributed to the paraformaldehyde contained in this fixative, which causes significantly slower penetration of Antigenfix into the tissues. Conversely, for both histochemical and molecular genetic studies, Antigenfix proved superior to formalin. Moreover, the results of our previous study showed that Antigenfix is a very good chemical agent for both short-term and long-term fixation for immunohistochemistry (Lenz et al. 2022a).

While many research groups have investigated the effect of different types of fixative solutions on pathological studies, only a few authors have addressed the effect of fixation time. Chung et al. (2018) did not find significant differences in the evaluation of morphological features when using formalin and alcohol fixatives (specifically 70% ethanol and BE70) in the period of 4 h to 6 months. These results are in contrast to our current study, which found formalin superior to alcohol fixative methacarn for histomorphological analysis. The present study is the first ever to analyze a fixation period of 1.5 to 369.5 days. Our results showed that long-term formalin fixation has no effect on histomorphological analysis, whereas methacarn fixation has a negative effect after only 64.5 days. In contrast to our current study, the study by Chung et al. (2018) did not demonstrate the fixation time and the type of fixative solution as significant factors affecting the concentration of the extracted DNA. Consistent with Chung et al. (2018) we report that DNA purity is higher when alcohol fixatives are used and also that DNA fragmentation is higher in formalin-fixed tissues compared to methacarn. More extensive fragmentation and lower purity of DNA is caused by the mechanism of formol's action on nucleic acids (i.e., formation of hydroxymethylene bridges, cross-linking of cytosine nucleotides and hydrolysis of N-glycosidic bonds), which leads to the formation of apurinic and apyrimidinic sites (Oboma and Ngokere 2018).

As for Bouin's fluid, our study has shown that this fixative is completely unsuitable for molecular genetic studies. The negative effect on nucleic acids is mainly caused by the acidic pH of this fixative solution. These results are consistent with those reported by Gatta et al. (2012). The study by Bonin et al. (2005) also found that Bouin was not suitable for molecular genetic studies. However, the authors of the latter study successfully amplified the 100 bp and 204 bp fragments. To improve Bouin's fixative for molecular genetic studies, Bonin et al. (2005) suggested the inclusion of an additional step

of DNA reconstruction. In our study, the failed amplification could be due to the very low concentration of DNA in the tissues fixed in Bouin's solution, which should reach at least 10 ng/µl according to the recommendation.

Only a limited number of studies have investigated the effect of tissue fixation on histochemical studies. For example, in the study by Howat and Wilson (2014), the effect of short-term 24-h fixation with different types of fixatives (specifically formalin, carnoy, methacarn, bouin, paraformaldehyde, 2% glutaraldehyde and zinc formalin) on Masson's trichrome staininig was analyzed. The authors reported the superiority of formalin and other aldehyde fixatives over alcohol fixatives. In our current study, we also obtained better results with formalin fixation compared to methacarn fixation for Gömöri reticulin stain. However, when using orcein staining, the results were completely comparable with both fixatives. Regarding the fixation time, we demonstrated that for both orcein and Gömöri staining, increasing time has no effect on the results of histochemical studies in methacarn-fixed tissues, while in formalin-fixed tissues, the decrease of all observed indices over time was significant.

Based on our results, it cannot be determined unequivocally whether aldehyde or alcohol fixatives are generally more suitable for histochemical studies. In line with Kap et al. (2011), we recommend optimizing staining protocols when choosing an alternative fixative for histochemical studies. Furthermore, not all histochemical studies can be approached in the same way. When choosing a suitable fixative, it is necessary to select an individual approach that takes into account both the type of fixative and the fixation time within that specific histochemical study. As demonstrated in our study, to preserve the quality of histochemical studies, alcohol fixatives are preferable to formalin for long-term fixation; alternatively, Antigenfix can be selected from the group of aldehyde fixatives. Finally, all types of fixatives (i.e. crosslinking, denaturing fixatives and fixatives combining both denaturing and cross-linking effects) can be used for short-term fixation when histochemical studies are required.

In conclusion, formalin confirmed its role as the most universal fixative overall. Due to its limitations, however, the selection of a suitable fixative should always be carried out strictly depending on the specific pathological study and the fixative time. Alcohol-based fixatives should be preferred for molecular genetic studies, and fixatives combining both denaturing and cross-linking effects may also be preferred over aldehyde fixatives for histochemical studies with long-term fixation. To date, an ideal fixative solution that is applicable to different tissue types and ancillary studies has not been found. Therefore, further research is needed in this area.

**Acknowledgement**

This study was supported by the project ITA of the University of Veterinary Sciences Brno (project no. 201011).

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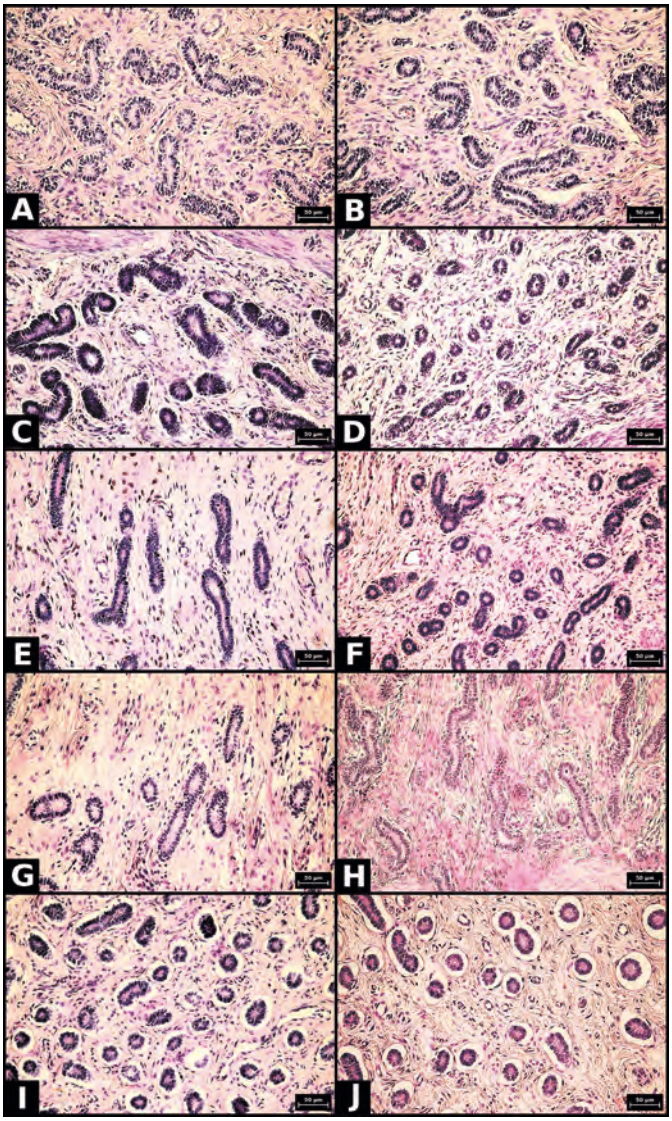


Fig. 1. Histomorphological analysis of porcine endometrial tissue samples fixed in formalin, Antigenfix, methacarn, Bouin's solution and Greenfix.

The main disadvantage of formalin fixation was the pericellular and cytoplasmic retraction observed during both short-term/1.5 (A) and long-term fixation/369.5 days (B). Using Antigenfix, pericellular and cytoplasmic retraction was mild and recognition of the cell nucleus was only satisfactory throughout the study, while distinction of individual cell types was good after 1.5 (C) and satisfactory after 369.5 days of fixation (D). The benefit of methacarn tissue fixation was the absence of pericellular and cytoplasmic retraction, while the other analyzed indicators were mostly assessed as satisfactory and decreased during the study from 1.5 days (E) to 369.5 days of fixation (F) The greatest advantage of Bouin's fixative was the very good recognition of nuclear details, however, the slides became more pink and red from 1.5 (G) to 369.5 days (H), which worsened the overall staining. The most limiting factor of Greenfix fixative during short-term/1.5 (I) and long-term fixation/369.5 days (J) was considerable pericellular retraction, which closely mimicked lymphovascular invasion (all images: hematoxylin and eosin staining, original magnification × 400, scale bar 50 μm).

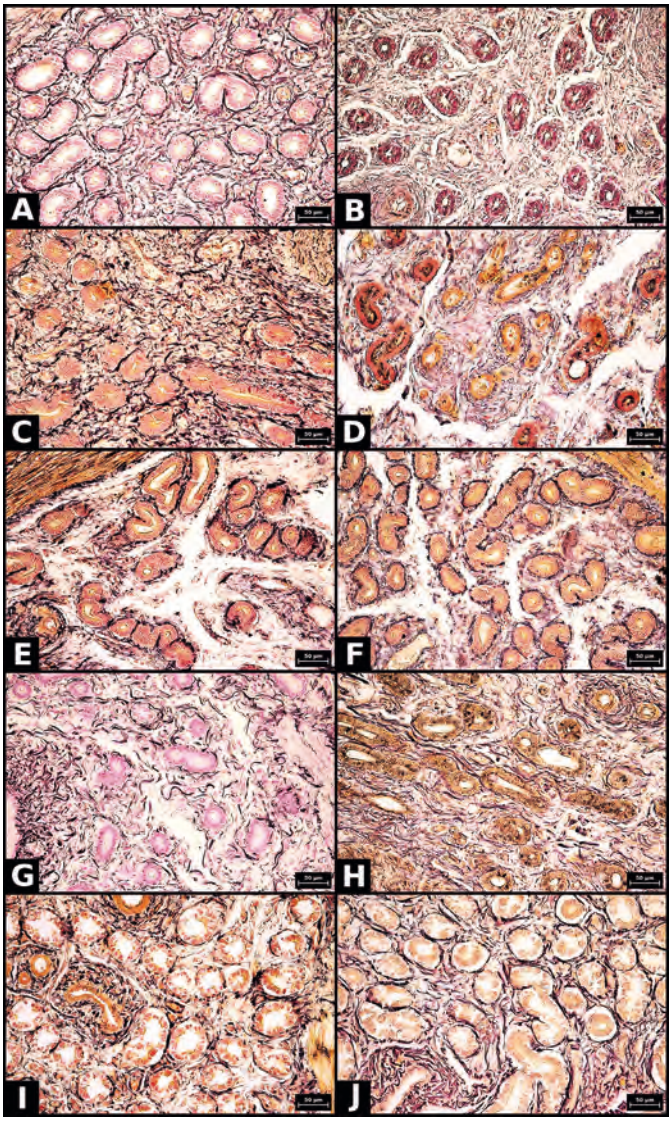


Fig. 2. Histochemical analysis of porcine endometrial tissue samples stained with Gömöri silver and fixed in formalin, Antigenfix, methacarn, Bouin's solution and Greenfix.

In formalin-fixed tissues, a significant decrease in the evaluation of all analyzed indicators was observed from very good after 1.5 days (A) to unsatisfactory after 189.5 days (B). Using Antigenfix, sharpness was assessed as very good throughout the study, while intensity and contrast decreased slightly from very good after 1.5 days (C) to good after 189.5 days of fixation (D). In methacarn-fixed tissues, staining intensity was assessed as good, contrast as very good, and sharpness as satisfactory during both short-term/1.5 (E) and long-term fixation /189.5 days (F). Using Bouin's fluid, all indicators were evaluated as very good after 1.5 days (G), and after 189.5 days of fixation (H) a significant decrease in contrast was noted, while intensity and sharpness decreased only slightly and were evaluated as good. For tissues fixed with Greenfix, all three indicators were evaluated as very good during short-term/1.5 (I) and long-term fixation /189.5 days (J) (all images: original magnification × 400, scale bar 50 μm).

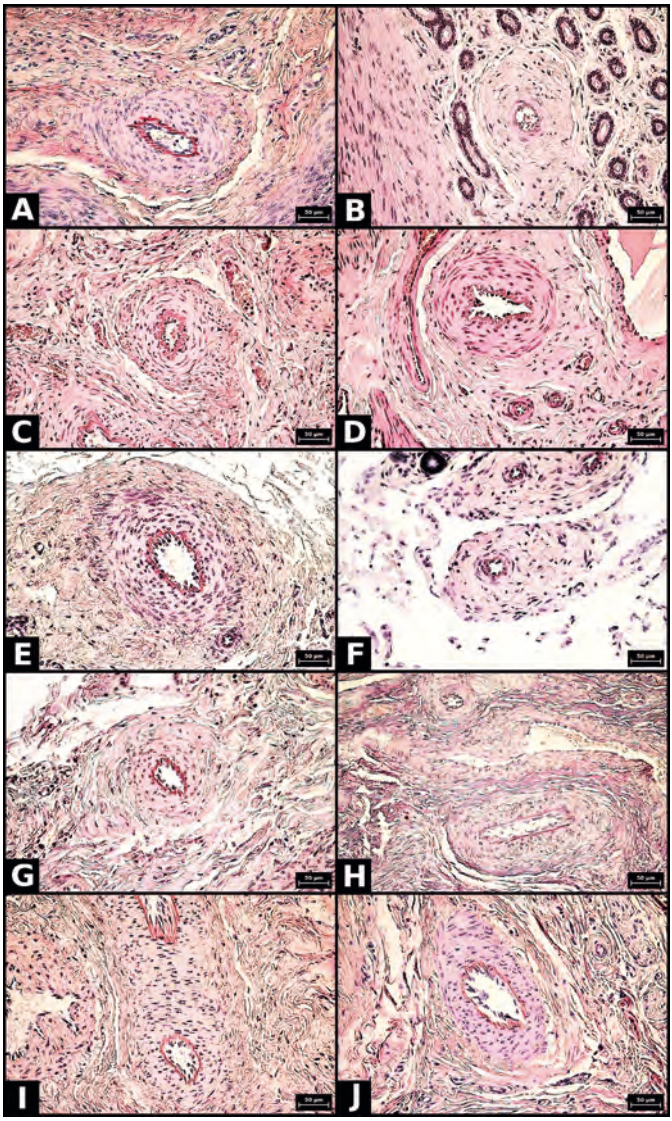


Fig. 3. Histochemical analysis of porcine endometrial tissue samples stained with orcein and fixed in formalin, Antigenfix, methacarn, Bouin's solution and Greenfix.

During formalin fixation, all three indicators significantly decreased from very good/good after 1.5 days (A) to unsatisfactory after 189.5 days of fixation (B). In Antigenfix-fixed tissues, intensity and sharpness were assessed as good and very good throughout the study, while contrast decreased from good after 1.5 days (C) to satisfactory after 189.5 days of fixation (D). Using methacarn fixative, intensity and contrast were assessed as good and sharpness as very good during both short-term/1.5 (E) and long-term fixation /189.5 days (F). For all indicators analyzed, which were assessed as very good after 1.5 days (G), a significant decrease to satisfactory was noted after 198.5 days of fixation (H) when fixed in Bouin's fluid. In Greenfix-fixed tissues, staining intensity was assessed as good throughout the study; contrast and sharpness slightly decreased from very good after 1.5 days (I) to good after 185.9 days of fixation (J) (all images: original magnification × 400, scale bar 50 μm).