Effects of different fixatives over different fixation times, including Antigenfix, on immunohistochemical studies

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Abstract

Tissue fixation is an essential step in the performance of ancillary studies, including immunohistochemistry. The aim of this study was to compare the effect of various fixatives and fixative times on immunohistochemistry (IHC) in bovine and porcine endometrium using progesterone receptors and SOX2 antibodies. Immunohistochemical staining with progesterone receptors and SOX2 was performed on tissue samples fixed in formalin, Antigenfix, Greenfix, Bouin's solution and methacarn at 1.5, 8.5, 15.5, 29.5, 64.5, 189.5, 249.5, 309.5 and 369.5 days of fixation. Formalin and Antigenfix proved to be the best fixative for both short-term and long-term fixation for IHC. Bouin's solution was partially applicable for short-term (24 h) fixation. Greenfix and methacarn were absolutely inappropriate fixatives for IHC (completely negative staining using methacarn). These results were obtained on an automated immunostainer using EDTA buffer pH 8.4 as the antigen retrieval solution. When the staining procedure was performed manually and citrate buffer with different pH values was used, weakly positive results were obtained with both progesterone receptors (pH 8 and 9) and SOX2 (pH 7.2); however, significantly lower staining quality was obtained using the methacarn fixative compared to aldehyde fixatives. The reported findings demonstrated the superiority of aldehyde fixatives (formalin and Antigenfix) over alcohol fixatives (methacarn) and fixatives combining both denaturing and cross-linking proteins (Greenfix and Bouin's solution) for IHC. Antigen retrieval-IHC using EDTA buffer was found to be excellent for aldehyde fixatives, but proved to be completely unsuitable for methacarn, Greenfix, and Bouin's solution.

Fixative solution, fixative period, EDTA buffer, immunohistochemistry, antigen retrieval technique

Tissue fixation is a key process of the pre-analytical phase. Inadequately or inappropriately fixed tissue leads to problems in tissue processing, microtomy, staining and performing ancillary studies (Fox et al. 1985).

Fixatives can broadly be divided into 4 groups based on the mechanism of their action. These are alcohol-based fixatives, oxidizing agents, aldehydes and the metallic group of fixatives (Matsuda et al. 2011). Alcohol-based fixatives, such as methacarn, are protein-denaturing agents. As a result of their action, water is removed from the free carboxyl, hydroxyl, amino, amido and imino groups of the proteins, which leads to destabilization of hydrophobic and hydrophilic bonds in proteins and consequently leads to changes in their secondary and tertiary structure (Baker 1958). Oxidizing agents and aldehydes act by cross-linking proteins. The most well-known and widely used aldehyde fixative in most laboratories is formaldehyde. The basic principle of formaldehyde fixation is the reaction between formalin and uncharged reactive amino groups (Buesa 2008). Another member of aldehyde fixatives is Antigenfix, a paraformaldehyde-based fixative solution. Bouin's fixative and Greenfix lead to both denaturing and cross-linking proteins. Bouin's fixative

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consists of picric acid, acetic acid, and formaldehyde, whereas Greenfix is a formaldehyde-free glyoxal- and ethanol-based fixative (Doyle and O'Leary 1992).

From an immunohistochemical point of view, it is important that the fixative maintains antigenic and structural integrity and also minimizes displacement, extraction or diffusion of antigen during subsequent tissue processing. Immunohistochemical staining is also affected by the fixation time. Overfixation may cause loss of antigenicity, while underfixation leads to insufficient tissue penetration of the fixative, resulting in incomplete cross-linking and denaturation proteins. Some of the disadvantages of tissue fixation, such as antigen masking caused by cross-linking fixatives, can be minimized using an antigen retrieval technique. Antigen retrieval, especially heat induced, breaks crosslinks caused by aldehyde-based fixatives and also helps to reduce the effect of prolonged fixation (Ramos-Vara 2005).

The aim of this study was to determine and compare the impact of various fixative agents and fixative times on immunohistochemistry (IHC) in bovine and porcine endometrium. Changes in nuclear expression were studied using progesterone receptors (PR), while the SOX2 antibody was employed to determine the effect on cytoplasmic expression.

Materials and Methods

Tissue specimens, fixation, and storage

A total of ten porcine and ten bovine endometrium obtained from hysterectomy specimens collected from healthy animals were included in this study. Tissue fragments of approximately 3.5 cm in length were dissected from the middle of one of the uterine horns of each sample. Subsequently, these fragments were divided into 5 equal parts; each part was placed in a separate container with one of the fixatives. A total of 5 fixatives were employed in our study, namely 10% neutral buffered formalin (NBF), Bouin's fixative solution, Greenfix, Antigenfix and methacarn (Table 1). Containers of the appropriate shape and size and a sufficient amount of fixative were provided for each sample (well-sealed and accurately labelled containers with a ratio of tissue volume to fixative volume of more than 1:20). Tissue sectioning of uterine samples was performed at 9 different time intervals: at 1.5, 8.5 (1 week), 15.5 (2 weeks), 29.5 (1 month), 64.5 (2 months), 189.5 (6 months), 249.5 (8 months), 309.5 (10 months) and 369.5 (12 months/1 year) days of fixation. The long-term storage was carried out in a designated storage area at room temperature (20–24 °C).

Further histoprocessing was performed according to standard procedures. In short, fixation was followed by dehydration (performed by increasing concentrations of alcohol) and incubation of sections in xylene leading to tissue clearing. This was followed by impregnation of the tissue blocks with paraffin. Finally, 4 μm thick tissue sections were cut from each block, placed on a glass slide, and stained with haematoxylin and eosin.

Representative tissue sections stained with haematoxylin and eosin were microscopically reviewed by a pathologist; no endometrial or myometrial pathology was found in any of the examined samples.

The study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Veterinary Sciences Brno.

Immunohistochemistry

Two antibodies were used for immunohistochemical analysis - PR (clone SP2, dilution 1:200, DCS, Hamburg, Germany) and SOX2 (polyclonal, dilution 1:75, Antibodies-online GmBH, Aachen, Germany). Immunohistochemical staining was performed on endometrial samples fixed in 5 different fixatives for varying fixation times (1.5, 8.5, 15.5, 29.5, 64.5, 189.5, 249.5, 309.5 and 369.5 days of fixation). Tissue sections of porcine endometrium were stained with PR, while SOX2 antibody was used only in bovine endometrial tissue samples. All immunohistochemical staining was performed on the automated stainer Benchmark Ultra (Ventana Medical System Inc., Oro Valley, AZ, USA) and the ultraView Universal DAB Detection Kit (Ventana Medical System Inc.) was used. The incubation times were 32 min and 16 min for PR and SOX2, respectively. An antigen retrieval technique was included for both antibodies employed: a heat-induced antigen retrieval was performed in CC1 buffer (Tris-ethylenediaminetetraacetic acid (EDTA)-based, pH 8.4) for 64 min (all samples) and for 20 min (only methacarn-fixed samples after 1.5 days of fixation) at 95 °C. In addition, immunohistochemical staining on methacarn-fixed tissues (after 1.5 days of fixation) was performed also manually using a heat-induced antigen retrieval in citrate buffer at different pH values (6.0, 7.2, 8.0 and 9.0) in water bath (95 °C) for 20 min.

The primary antibodies were visualised using hydrogen peroxide substrate and 3,3'- diaminobenzidine tetrahydrochloride (DAB) chromogen. External positive controls were prepared for both antibodies (human endometrium for PR and human spleen for SOX2). Negative controls were prepared by incubating samples without a primary antibody. Evaluation of immunohistochemical analysis was performed using a uniform microscope and camera setting (Olympus BX53 microscope and Promican 3-3CC camera).

Table 1. Summary of fixatives used for tissue fixation in this study.

Fixative solution	Composition	pH	Manufacturer (city, country)	Mechanism of action
NBF	Formaldehyde, methanol, sodium phosphate monobasic, sodium phosphate dibasic, deionized water	7.0-7.4	Diapath S.p.A. (Martinengo, Italy)	Cross-linking
Bouin's fixative	Formaldehyde, acetic acid, icric acid	< 2	Dr. Kulich Pharma s.r.o. (Hradec Králové, Czech Republic)	Cross-linking/ denaturing
Greenfix	Ethanedial solution, ethyl alcohol, non-toxic preserving agents	< 5	Diapath S.p.A. (Martinengo, Italy)	Cross-linking/ denaturing
Antigenfix	Paraformaldehyde, monobasic, sodium phosphate sodium phosphate dibasic	7.0-7.4	Diapath S.p.A. (Martinengo, Italy)	Cross-linking
Methacarn	Methanol, chloroform, acetic acid	-	Methanol - Dr. Kulich Pharma s.r.o. (Hradec Králové, Czech Republic) Chloroform and acetic acid - Penta s.r.o. (Prague, Czech Republic)	Denaturing

NBF - 10% neutral buffered formalin; pH - potential of hydrogen.

Table 3. Immunohistochemical expression of progesterone receptors in porcine endometrium fixed in methacarn and Greenfix.

Fixative period (days)	Methacarn		Greenfix	
	% of positive cells	Intensity of staining	% of positive cells	Intensity of staining
1.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**37** (40, 35, 35, 35, 40, 40, 40, 35, 35, 35)	W
8.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
15.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
29.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
64.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
189.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
249.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
309.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
369.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N

W - weak; N - negative reaction.

Evaluation of immunostaining

Nuclear staining for PR in porcine endometrium and cytoplasmic staining for SOX2 in bovine endometrium was considered positive. Only endometrial glandular cells were evaluated (not endometrial stroma). The percentages of marker positive cells were evaluated using a light microscope at a × 200 magnification. At least eight foci of bovine and porcine endometrium were analysed and the percentage of marker positive cells of the covered area was determined. A positive cut-off value of greater than 1% of cells staining was selected. The intensity of immunoreaction was also investigated, distinguishing between weak, moderate, strong and extra strong staining.

Table 2. Immunohistochemical expression of progesterone receptors in porcine endometrium fixed in NBF, Bouin's solution and Antigenfix.

Fixative period (days)	NBF		Bouin's solution		Antigenfix	
	% of positive cells	Intensity of staining	% of positive cells	Intensity of staining	% of positive cells	Intensity of staining
1.5	**92.5** (90, 90, 95, 90, 95, 90, 90, 95, 95, 95)	S	**8.5** (10, 10, 5, 10, 10, 5, 10, 10, 10, 5)	W	**94** (90, 95, 90, 95, 95, 90, 95, 100, 95, 95)	S
8.5	**92** (90, 95, 90, 95, 90, 90, 90, 95, 95, 90)	S	**0** (0,0,0,0,0,0,0,0,0,0)	N	**83.5** (80, 75, 80, 80, 90, 85, 85, 90, 80, 90)	S
15.5	**92** (90, 95, 90, 90, 90, 95, 95, 90, 95, 90)	S	**0** (0,0,0,0,00,0,0,0,0)	N	**73.5** (70, 75, 75, 70, 75, 80, 75, 70, 70, 75)	S
29.5	**91.5** (90, 90, 95, 95, 90, 90, 95, 90, 90, 90)	S	**0** (0,0,0,0,00,0,0,0,0)	N	**70** (65, 70, 75, 70, 75, 70, 70, 70, 65, 70)	S
64.5	**91** (95, 90, 90, 90, 90, 95, 90, 90, 90, 90)	S	**0** (0,0,0,0,0) 0,0,0,0,0	N	**69** (60,70, 70, 70, 70, 75, 70, 70, 65, 70)	M
189.5	**66** (70, 70, 65, 60, 65, 70, 65, 60, 65, 70)	W	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0*** (1,0,0,3,0,0,0,2,0,0)	N
249.5	**63.5** (65, 65, 65, 60, 65, 65, 60, 65, 60, 65)	W	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
309.5	**51.5** (50, 55, 50, 50, 55, 50, 55, 50, 50, 50)	W	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
369.5	**31** (30, 30, 35, 30, 35, 30, 25, 30, 35, 30)	W	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N

S - strong; W - weak; M - moderate; N - negative reaction; NBF - 10% neutral buffered formalin.
* a cut-off value of >1% of marker positive cells was considered to represent a positive result

Table 4. Immunohistochemical expression of SOX2 in bovine endometrium fixed in NBF, Bouin's solution and Antigenfix.

Fixative period (days)	NBF		Bouin's solution		Antigenfix	
	% of positive cells	Intensity of staining	% of positive cells	Intensity of staining	% of positive cells	Intensity of staining
1.5	**99**(100, 95, 100, 100, 100, 100, 95, 100, 100, 100)	ES	**97** (100, 95, 100, 100, 95, 95, 90, 100, 95, 95)	M	**99** (100, 100, 95, 100, 100, 100, 100, 100, 100, 95)	ES
8.5	**99** (100, 95, 100, 100, 95, 100, 100, 100, 100, 100)	S	**0** (0,0,0,0,0,0,0,0,0,0)	N	**98.5** (95, 100, 100, 100, 100, 100,95,100, 100, 95)	ES
15.5	**98.5** (100, 100, 95, 100, 100, 100, 95, 100, 100, 95)	S	**0** (0,0,0,0,0,0,0,0,0,0)	N	**98.5** (95, 100, 100, 100, 95, 100, 100, 100, 100, 95)	S
29.5	**98.5** (100, 95, 100, 100, 95, 100, 100, 100, 100, 95)	S	**0** (0,0,0,0,0,0,0,0,0,0)	N	**98.5** (100, 95, 100, 95, 100, 100, 100, 100, 100, 95)	S
64.5	**98.5** (100, 100, 95, 100, 100, 100, 100, 95, 100, 95)	S	**0** (0,0,0,0,0,0,0,0,0,0)	N	**98.5** (95, 100, 100, 95, 100, 100, 95, 100, 100, 100)	S
189.5	**98** (95, 100, 100, 100, 95, 100, 95, 100, 100, 95)	M	**0** (0,0,0,0,0,0,0,0,0,0)	N	**98.5** (95, 100, 100, 100, 95, 100, 100, 95, 100, 100)	S
249.5	**98** (95, 100, 100, 100, 95, 100, 95, 100, 100, 95)	M	**0** (0,0,0,0,0,0,0,0,0,0)	N	**98** (95, 100, 100, 100, 95, 100, 95, 100, 100, 95)	S
309.5	**98** (95, 100, 100, 100, 95, 100, 95, 100, 100, 95)	M	**0** (0,0,0,0,0,0,0,0,0,0)	N	**98** (95, 100, 100, 100, 95, 100, 95, 100, 100, 95)	S
369.5	**97.5** (95, 100, 95, 100, 95, 100, 95, 100, 100, 95)	M	**0** (0,0,0,0,0,0,0,0,0,0)	N	**98** (95, 100, 100, 100, 95, 100, 95, 100, 100, 95)	S

ES - extra strong; S - strong; M - moderate; N - negative reaction; NBF - 10% neutral buffered formalin.

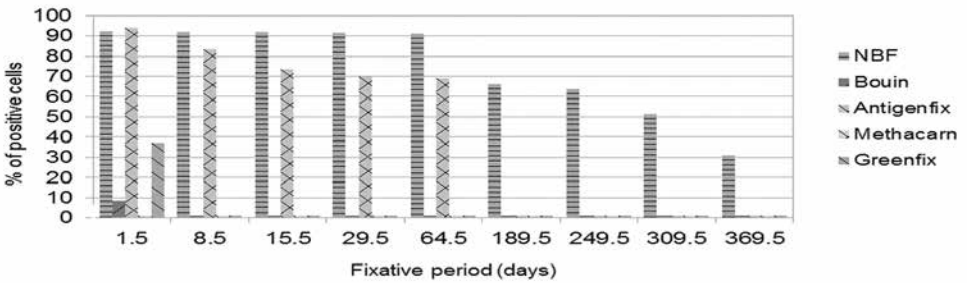

Fig. 1. Immunohistochemical expression of PR in porcine endometrium
NBF - 10% neutral buffered formalin; PR - progesterone receptors

Statistical analysis

The differences were compared using *t*-test because Shapiro-Wilks tests were not able to reject the normality of the data. Differences in various time intervals were compared using paired *t*-test, whereas differences between the individual fixatives were compared using two-sample *t*-test.

Results

PR expression in porcine endometrium

Results of the analysis of the PR expression in porcine endometrium are presented in Tables 2, 3 and Fig. 1.

NBF

After 1.5–64.5 days of fixation, strong nuclear expression of PR was demonstrated in more than 91% of glandular cells (Plate IV, Fig. 2A–E). After 189.5 days of fixation, the intensity of staining was evaluated as moderate and the percentage of positive cells decreased significantly to approximately 66% (Fig. 2F). Another significant decrease was detected over all remining time intervals (63.5% positivity after 249.5 days; 51.5% positivity after 309.5 days and 31% positivity after 369.5 days of fixation) ($P < 0.001$), in which the intensity of staining was evaluated as weak (Fig. 2G–I).

Antigenfix

In Antigenfix-fixed tissues, strong PR expression was observed in 94% of cells after 1.5 days of fixation (Plate II, Fig. 3A). After 8.5 days, the staining was still strong, but the percentage of positive cells decreased slightly to 83.5% (Fig. 3B). A significant decrease was observed during the following time periods: strong staining in 73.5% of glandular cells after 15.5 days and strong to moderate staining in 70% and 69% of cells after 29.5 and 64.5 days of fixation ($P < 0.001$) (Fig. 3C–E). Over the remaining time intervals (189.5, 249.5, 309.5 and 369.5 days), the immunoreaction was negative (Fig. 3F). After long-term fixation (more than 189.5 days), significant differences were found between NBF and Antigenfix ($P < 0.001$).

Bouin's fixative

In Bouin's fixative solution, 8.5% of the cells were stained after 1.5 days of fixation and the intensity of staining was evaluated as weak (Plate V, Fig. 4A). At other time intervals, the staining was completely negative (Fig. 4B). When comparing Bouin's fixative with NBF and Antigenfix, significant differences in short-term fixation (1.5 days) were observed ($P < 0.001$).

Greenfix

After 1.5 days of tissue fixation in Greenfix, 37% of glandular cells showed weak PR expression (Fig. 4C). In the other fixative periods, immunohistochemical staining was negative (Fig. 4D). Regarding short-term fixation (1.5 days), significant differences were found between Greenfix and Bouin's fixative, as well as between NBF and Greenfix and Bouin's fixative ($P < 0.001$).

Methacarn

Immunohistochemical staining with PR in methacarn-fixed tissues was completely negative over all fixative times (regardless of heating time) (Fig. 4E).

SOX2 expression in bovine endometrium

Results of the analysis of the SOX2 expression in bovine endometrium are presented in Tables 4, 5 and Fig. 5.

Table 5. Immunohistochemical expression of SOX2 in bovine endometrium fixed in methacarn and Greenfix.

Fixative period (days)	Methacarn		Greenfix	
	% of positive cells	Intensity of staining	% of positive cells	Intensity of staining
1.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
8.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
15.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
29.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
64.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
189.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
249.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
309.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N
369.5	**0** (0,0,0,0,0,0,0,0,0,0)	N	**0** (0,0,0,0,0,0,0,0,0,0)	N

N - negative reaction.

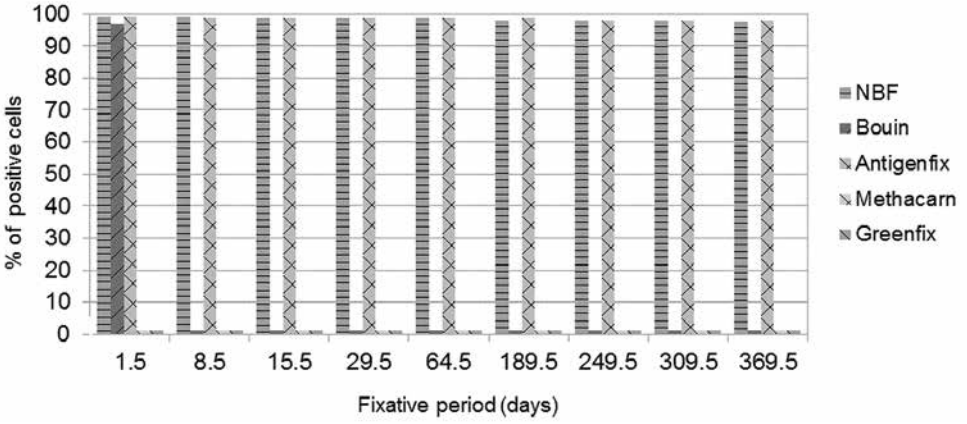

Fig. 5. Immunohistochemical expression of SOX2 in bovine endometrium
NBF - 10% neutral buffered formalin

NBF

The intensity of staining with SOX2 antibody using NBF was classified as extra-strong after 1.5 days, strong after 8.5, 15.5, 29.5 and 64.5 days and moderate after 189.5, 249.5, 309.5 and 369.5 days of fixation (Plate V, Fig. 6A–I). The percentage of SOX2 positive cells was greater than 97% over all fixation intervals (no perceptible differences).

Antigenfix

Similar results were found in antigenfix-fixed tissues as in NBF. No differences in the percentage of SOX2 positive cells were noted between 1.5 and 369.5 days of fixation – immunopositivity in 98–99% of glandular cells. Extra-strong staining was observed after 1.5 and 8.5 days; over all remaining time periods the intensity of the reaction decreased to strong (Plate VI, Fig. 7A–I). In terms of short-term and long-term fixation, the results of immunohistochemical staining with SOX2 antibody using antigenfix and NBF are completely comparable. The only difference was the intensity of the staining after more than 189.5 days (moderate for NBF vs. strong for Antigenfix); however, this finding has no impact on routine practice because extra strong and strong staining is always classified as a positive reaction in the immunohistochemical analysis.

Bouin's fixative

After 1.5 days of fixation in Bouin's solution, moderately intense immunostaining of SOX2 was detected in 97% of glandular cells (Plate V, Fig. 4F). Complete immunonegativity was noted over all other time periods (Fig. 4G).

Greenfix and Methacarn

No SOX2 positivity was detected in tissues fixed in Greenfix or methacarn for any fixation period (Fig. 4H–I).

Analysis of PR and SOX2 in porcine and bovine endometrium using heat-induced antigen retrieval in citrate buffer at different pH after 1.5 days of fixation in methacarn

Weak nuclear expression of PR using citrate buffer pH 8 (Plate VI, Fig. 8A) and moderate nuclear expression using citrate buffer pH 9 were found in more than 90% of glandular cells (Fig. 8B). Staining with PR using citrate buffers pH 6 and 7.2 yielded negative results. For the SOX2 antibody, weak and sporadic cytoplasmic expression was detected only using citrate buffer pH 7.2 (Fig. 8C); staining using other pH values was completely negative.

Discussion

Several studies analysed the impact of the fixation time and the type of fixative on IHC. In a study by Paavilainen et al. (2010), a stronger intensity of immunostaining after short-term 24-h fixation using aldehyde-based fixatives was found compared to alcohol fixatives. These results are fully consistent with the results of the presented study. In addition, we found the same results even after long-term fixation.

Compared to our study, there are conflicting results in the literature regarding the effect of methacarn tissue fixation on IHC. According to our findings, methacarn is completely unsuitable for IHC. Expression was not observed with any of the markers used at any fixative time, although the manufacturer states that methacarn is applicable to immunohistochemical studies. One of the mentioned advantages of methacarn is the ability to protect the antigenic sites of the tissues, which, according to the recommendations, also makes it possible to omit the unmasking of antigens. Milcheva et al. (2013) do not agree with this statement. They demonstrated a completely negative immunoreaction of methacarn-fixed tissues

when antigen unmasking was omitted. Negative immunohistochemical staining during fixation in methacarn may be due to denaturation of the antigen-binding sites. Therefore, the authors recommend that antigen unmasking be performed at all times and reported that the non-specific background staining caused by the secondary antibody was reduced by this technique, and that more intense immunohistochemical staining was obtained after methacarn fixation than after formalin fixation. Regarding the antigen retrieval method, the authors used citrate buffer pH 6.2 at sub-boiling temperature for 1, 5, and 10 min. In another immunohistochemical study, Delfour et al. (2006) investigated the effect of methacarn fixation on oestrogen receptors alpha (ESR1), HER2 and PR expression in breast tumour. The authors demonstrated similar staining results using formalin and methacarn fixatives after a short-term 24-h fixation. They mentioned the need to optimize the antigen retrieval technique for both fixatives. In their study, antigen retrieval was performed in a water bath using EDTA or citrate buffer, but no further details on this technique were provided in the paper. Interestingly, Mitchel et al. (1985) reported methacarn to be the best fixative to preserve tissue antigenicity. In their study, modified methacarn was compared with NBF, Bouin, Carnoy and B-5. The modification of methacarn consisted of the substitution of chloroform for inhibisol. The authors reported that such modification did not affect tissue antigenicity, because the main compound of the methacarn solution providing tissue fixation is methanol. In their study, the proteolytic-induced antigen retrievel technique using the pronase enzyme was used for all samples (regardless of the type of fixative). In our study, all immunohistochemical tests were performed on the automated immunostainer Benchmark Ultra. In this machine, the staining process takes approximately 3 h and involves a total of 12 steps, one of which is the unmasking of antigens (this step is referred to in the protocol as cell conditioning). Unlike all of these studies, we did not demonstrate a positive immunohistochemical reaction in methacarn-fixed tissues, although the antigen retrieval (unmasking) technique was included. To determine the cause of these conflicting results, immunohistochemical staining of tissue samples fixed in methacarn (fixative period of 1.5 days) was performed manually using a citrate buffer at various pH values as the antigen retrieval solution. Regarding PR, similar results were obtained for formalin-fixed tissues as for methacarn-fixed tissues using citrate buffer pH 9. In contrast, only weak sporadic SOX2 expression was detected in less than 1% of cells using citrate buffer pH 7.2 (compared to diffuse extra strong SOX2 expression using EDTA buffer pH 8.4 in formalin-fixed tissues). Given these results, we concluded that antigen retrieval-immunohistochemistry (AR-IHC) is pH-dependent and that AR-IHC staining using EDTA buffer is unsuitable for methacarn-fixed tissues. In searching the literature, no publication describing a negative effect of methacarn tissue fixation on IHC was found.

Using Bouin's fixative solution, we found sporadic weak nuclear expression of PR and diffuse moderately intense cytoplasmic expression of SOX2. Staining was detected only after 24 h of fixation, other time intervals were completely negative. Thus, our study showed poor antigen preservation using Bouin's fixative, even when the antigen retrieval technique was included. Some investigators have arrived at the same conclusion (Williams et al. 1997). Bouin's fixative was also included in a study by Mitchel et al. (1985), which demonstrated that some of the antibodies employed showed a significant decrease in immunoreactivity when using Bouin's solution. For example, EMA expression in formalin-fixed tissue was the same as in tissues fixed in Bouin's solution, while collagen IV expression was significantly reduced using Bouin's fixative compared to formol. In one additional immunohistochemical study by Gatta et al. (2012), staining with PAN-cytokeratin (PAN-CK), CD31, Ki-67, S100, CD68 and smooth muscle actin (SMA) antibodies was performed on various tissue types fixed in 6 different fixatives (NBF, Bouin, Hollande, Greenfix, UPM, CyMol) for 24 h. The authors found both a lower intensity of immunostaining and the percentage of PAN-CK, CD31, and Ki-67 positive cells in

tissues fixed in Bouin's solution compared to formalin fixative. In contrast, there was an inverse correlation between formalin and all other fixatives in the CD68 expression. In summary, reduced effectiveness of antigen retrieval has been demonstrated by these and other studies (Allison and Best 1998; Miller et al. 1999) when using fixatives other than NBF (including Bouin's solution), which is consistent with the present study. Based on the results and available histological images of these studies (including ours), it can also be concluded that the cytoplasmic staining is more resistant to the effects of fixation compared to nuclear staining. However, contradictory conclusions were published by Matsuda et al. (2011), who reported that differences in immunoreactivity depend only on factors affecting fixation and not on the cellular localization of the antigen.

Apart from our study, only Gatta et al. (2012) investigated the effect of Greenfix fixation on immunohistochemical staining with conflicting results. After 24 h of fixation, we found a weak focal PR expression, whereas the authors of the above mentioned study reported comparable intensity of staining and percentage of marker positive cell in Greenfix- and formalin-fixed tissues. To our knowledge, our study is the first to document a completely negative staining after more than 24 h of Greenfix fixation. We therefore believe that Greenfix is not a suitable fixative where immunohistochemical studies are required.

To our knowledge, our study is also the first to address the effect of Antigenfix tissue fixation on IHC. Antigenfix achieved comparable results with formalin and therefore appears to be a very good chemical agent for both short-term and long-term fixation for IHC. Thus, Antigenfix is another member of the aldehyde fixatives suitable for immunohistochemical studies.

In one recent study, Chung et al. (2018) investigated and compared the effect of formalin and ethanol-based fixatives on IHC. Surprisingly, they reported that the intensity of staining and the number of marker-positive cells were relatively time-independent in ethanol and BE70, while a radical decrease of both indicators was noted after only 1 week of fixation in NBF. The authors concluded that for the purposes of long-term fixation, alcohol-based fixatives are more suitable for immunohistochemical analysis than cross-linking fixatives. We found several differences compared to this study. In particular, we have demonstrated contradictory findings with regard to long-term NBF fixation. Furthermore, we have shown that the alcohol-based fixative methacarn does not support immunohistochemical staining methods. However, it is difficult to compare the two studies in detail because the study by Chung et al. (2018) lacks information about the type of antigen retrieval solution.

When evaluating the effect of different types of fixatives on IHC, it is important to know the characteristics of the antigen under investigation, one of the most important of which is its cellular localization. Structural proteins, such as histones, intermediate filaments or some membrane proteins, are resistant to factors of specimen handling (excision) and tissue processing (formalin fixation, dehydration) and thus remain in their original cellular compartment. In contrast, water-soluble proteins and small peptides may alter their intracellular localization during tissue processing or due to a pathological condition such as inflammation (McKeever 2012). Modified cellular localization could induce changes in the structural integrity of proteins, which could significantly affect their resistance to various types of fixatives. We believe that this mechanism could be one of the causes of negative immunohistochemical results when using various fixatives. Therefore, we concluded that immunohistochemical analysis depends on both the cellular localization of the antigen and the parameters of fixation.

To summarize, NBF proved to be the best fixative for both short-term and long-term fixation for immunohistochemical studies. Similar results were obtained with Antigenfix fixative (especially for short-term fixation). Bouin's solution was partially applicable for short-term (24-h) fixation, but was far from ideal for this special staining method. Greenfix and methacarn were absolutely inappropriate fixatives for IHC using EDTA buffer as the antigen retrieval solution. NBF and Antigenfix were equivalent fixatives for the

visualisation of both nuclear and cytoplasmic antigens if the fixation period did not exceed 2 months. With a longer fixation time, cytoplasm has been shown to be more resistant to the effects of chemical fixation compared to the nuclear compartment.

The present study also points out several novel findings that have not yet been published in scientific literature. Our work was the first to demonstrate the negative effect of methacarn tissue fixation on immunohistochemical studies, although the antigen retrieval technique was part of the staining process. We have also shown that long-term fixation with Greenfix does not support immunohistochemical studies. For the first time, the effect of Antigenfix fixation on IHC was analysed.

In conclusion, the reported findings demonstrate the superiority of cross-linking fixatives over alcohol fixatives for immunohistochemical studies. Formalin confirmed its role of a satisfactory fixative, provided that an effective antigen retrieval technique is part of the immunohistochemical staining process. Equivalent immunohistochemical results were obtained with Antigenfix, another member of the cross-linking fixatives that can be considered as an alternative to formalin. With strict adherence to standard procedures of specimen handling and histoprocessing and the use of EDTA buffer as the antigen retrieval solution, denaturing fixatives and fixatives combining both denaturing and cross-linking proteins have been shown to be completely unsuitable for immunohistochemical analysis. The results of our work emphasize the need to optimize the antigen retrieval technique, especially depending on the type of antigen retrieval solution and the pH used.

To the authors' knowledge, this is the first study of its kind in the veterinary literature in which tissue samples of farm animals were employed.

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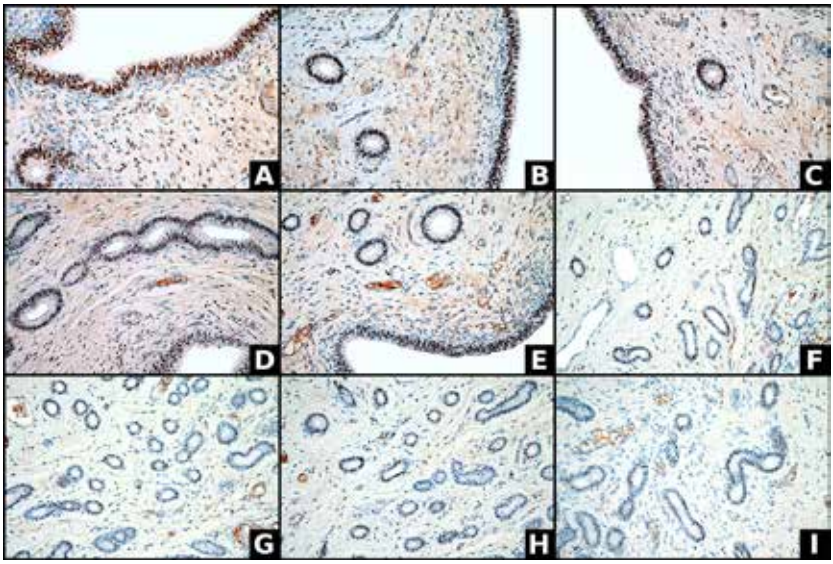

Fig. 2. Immunohistochemical expression of PR in formalin-fixed tissue samples
Strong nuclear expression of PR in more than 91% of glandular cells after 1.5 (A), 8.5 (B), 15.5 (C), 29.5 (D) and 64.5 days (E) of fixation. Moderate nuclear PR expression in approximately 66% of glandular cells after 189.5 days of fixation (F). Weak nuclear PR expression in approximately 63.5%, 51.5% and 31% of glandular cells after 249.5 (G), 309 (H) and 369.5 (I) days of fixation (all images: immunohistochemistry, original magnification × 400).

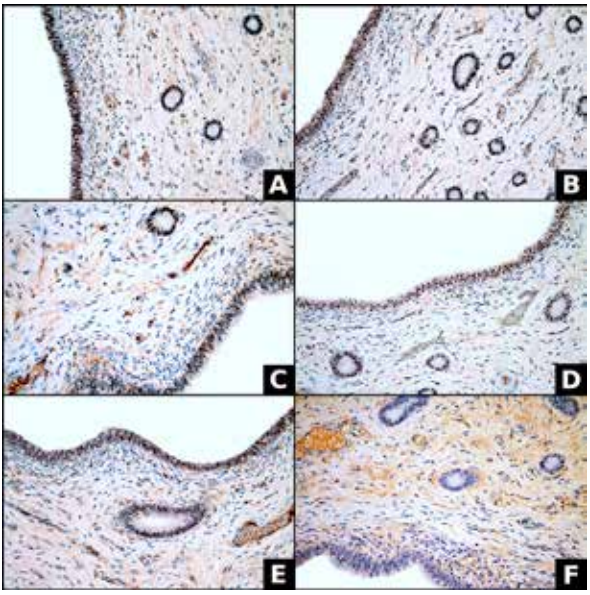

Fig. 3. Immunohistochemical expression of PR in Antigenfix-fixed tissue samples.
Strong nuclear expression of PR in approximately 94%, 83.5% and 73.5% of glandular cells after 1.5 (A), 8.5 (B) and 15.5 days (C) of fixation. Moderate to strong PR expression in approximately 70% and 69% of cells after 29.5 (D) and 64.5 days (E) of fixation. Expression of PR in less than 1% of glandular cells is considered to represent a negative result (positive cut-off value > 1%) (F) (all images: immunohistochemistry, original magnification × 400).

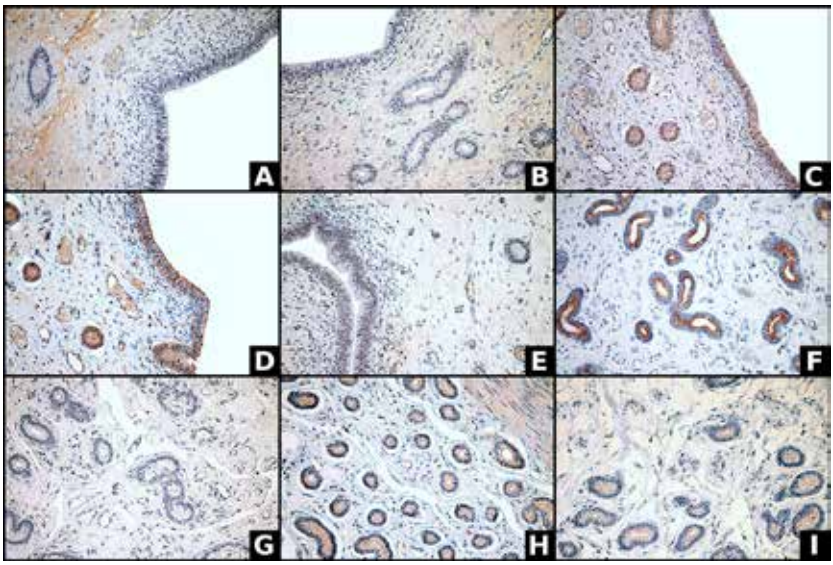

Fig. 4. Immunohistochemical expression of PR and SOX2 in tissue samples fixed in Bouin's solution, Greenfix and methacarn.
Weak nuclear expression of PR in approximately 8.5% of glandular cells after 1.5 days (A) and negative staining after 8.5 days of fixation using Bouin's solution (B). Weak nuclear expression of PR in approximately 37% of glandular cells after 1.5 days (C) and negative nuclear staining after 8.5 days of fixation using Greenfix (D). Negative staining for PR after 1.5 days of fixation using methacarn (E). Diffuse moderately intense cytoplasmic expression of SOX2 after 1.5 days (F) and negative staining after 8.5 days (G) of fixation using Bouin's solution. Negative staining for SOX2 after 1.5 days of fixation using greenfix (H) and methacarn (I) (all images: immunohistochemistry, original magnification × 400).

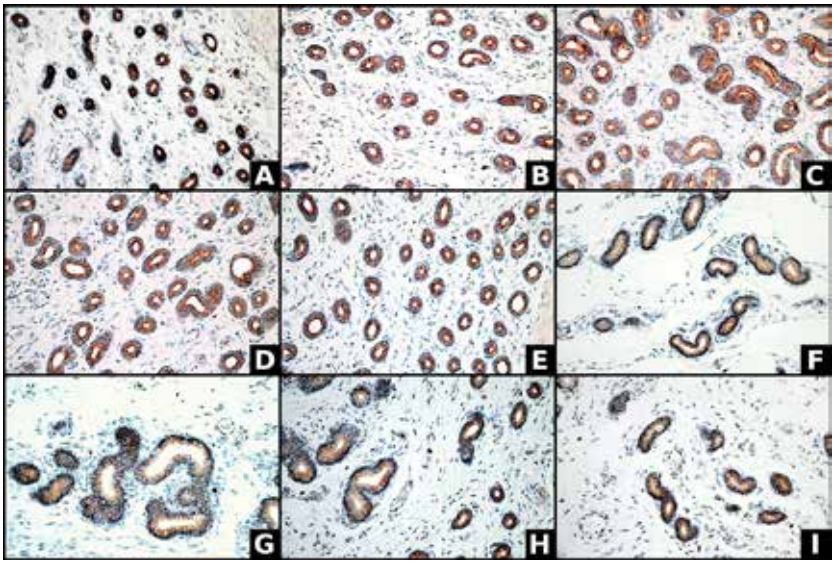

Fig. 6. Immunohistochemical expression of SOX2 in formalin-fixed tissue samples.
Diffuse extra-strong cytoplasmic SOX2 expression after 1.5 days (A), diffuse strong cytoplasmic expression after 8.5 (B), 15.5 (C), 29.5 (D) and 64.5 days (E) and diffuse moderate cytoplasmic expression after 189.5 (F), 249.5 (G), 309.5 (H) and 369.5 days (I) of fixation (all images: immunohistochemistry, original magnification × 400).

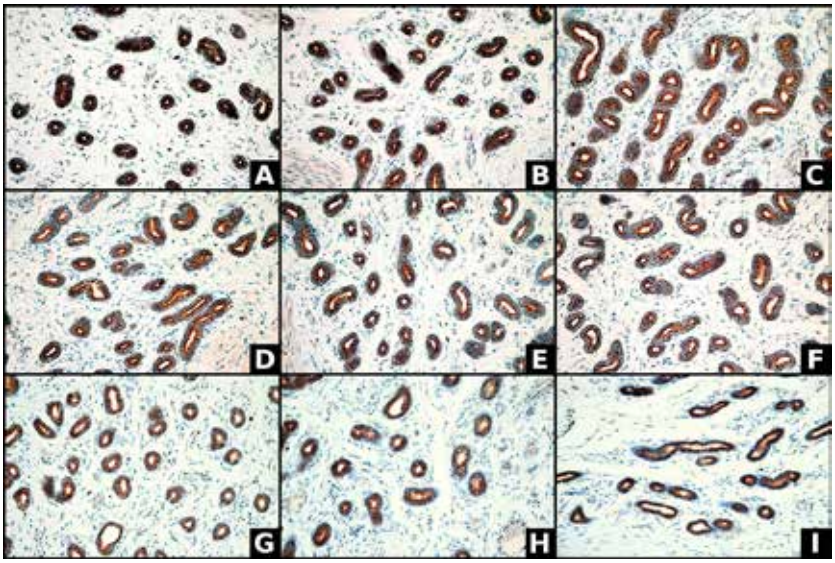

Fig. 7. Immunohistochemical expression of SOX2 in Antigen-fixed tissue samples.
Diffuse extra-strong cytoplasmic SOX2 expression after 1.5 (A) and 8.5 days (B) and diffuse strong cytoplasmic expression after 15.5 (C), 29.5 (D), 64.5 days (E), 189.5 (F), 249.5 (G), 309.5 (H) and 369.5 days (I) of fixation (all images: immunohistochemistry, original magnification × 400).

Fig. 8. Immunohistochemical expression of PR and SOX2 after short-term (1.5 days) methacarn fixation using heat-induced antigen retrieval in citrate buffer with pH values ranging from 7.2 to 9.
Weak nuclear expression of PR in more than 90% of glandular cells using citrate buffer pH 8 (A). Moderate nuclear expression of PR in more than 90% of glandular cells using citrate buffer pH 9 (B). Weak cytoplasmic expression of SOX2 using citrate buffer pH 7.2 (C) (all images: immunohistochemistry, original magnification × 400).