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RESEARCH ARTICLE

Influence of fixation method and duration of archiving on immunohistochemical staining intensity in embryonic and fetal tissues

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ABSTRACT

Introduction: In this study we detail the effect of different fixation agents and the duration of storage has on the immunohistochemical staining positivity of samples of archival embryonic and fetal tissues.
Materials and methods: The samples were stained by indirect two-step immunohistochemistry (IHC) method for Ki-67, cyclin A and β-actin.
Results: Irrespective of the length of tissue archiving, tissue fixation with 10% neutral buffered formalin had better IHC intensity results in all cases when compared to methacarn-fixed tissues. In the case of β-actin, this difference was statistically significant, while differences in Ki-67 and cyclin A were not. The second aspect studied was which effect tissue block archiving duration has on the IHC reactivity. We demonstrated a statistically significant decrease in IHC positivity for all studied antigens between the samples that were archived for 10–19 or 20–45 years, regardless the fixative solution.
Conclusion: To the best of our knowledge, the influence that the duration of tissue block archiving has on IHC positivity in human embryo and fetal tissue material has not yet been studied. Although the causes of the IHC positivity decline in archived tissue blocks are not well understood, a possible decrease in IHC over time should be considered, particularly in retrospective studies.

1. Introduction

Currently, immunohistochemical detection of a wide range of antigens is a method routinely used by histopathological laboratories. Immunohistochemistry (IHC) results are increasingly utilized not only for routine diagnostics but also for their potential predictive and prognostic significance (Tashima et al., 2015; Wang et al., 2016). However, it is known that IHC positivity can be affected by a number of factors, such as tissue procurement, the size of the sample, time of processing, the type of fixation used, length of fixation, method of processing, the type of antibody used, length of sample archiving and a number of others.

The effect of different types of fixatives was investigated by many authors (Bos et al., 2000; Dowsett et al., 2011; Mengel et al., 2002; Paavilainen et al., 2010). These authors agreed that among fixative agents, 10% neutral buffered formalin (pH neutral 4% formaldehyde) is considered as a "gold standard" allowing correct tissue preservation and IHC detection. Nevertheless, a number of other fixative substances, especially alcohol-based fixatives including methacarn, have been sequentially tested with the aim of achieving better IHC tissue positivity, and to avoid unnecessary exposure to formaldehyde, which is inherently highly toxic. The studies conducted by Mengel et al. and Dowsett et al. led to the same result, that in case of Ki-67, a fixation with agents other than formalin reduces IHC staining. Bos and co-athors studied the effect of the fixatives used on the immunopositive properties of TGFβ1, TGFβ3, IGF1, IGF2 and FGF2 in cartilage tissue. Best results were obtained by using formaldehyde-based fixatives, while a reduction of immunoreactivity and discrepancies in subcellular localization in alcohol-fixed tissues was also observed (Bos et al.,

Abbreviations: FGF, fibroblast growth factor; IGF, insuline-like growth factor; IHC, immunohistochemistry; IUD, intrauterine development; TGF, transforming growth factor.

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2000). Paavilainen et al. (2010) evaluated the effect of seven different fixatives (Fine FIX, Glyo-fixx, HOPE, NBF, NEO-FIX, ZBF and ZnF) on the immunopositivity of antigens with different subcellular localization and biological function in tonsil, kidney, liver, colon, placenta, thyroid, prostate, and stomach tissues, and also at the level of tissue cultures and protein lysates. Moreover, Warmington et al. reported a lack of tissue fixation and a need of heat-induced antigen retrieval in different tissues fixed by ethanol-based fixatives (Warmington et al., 2000). Conversely, Delfour et al. did not prove a difference in the detection of estrogen and progesterone receptors and Her2 in formalin- and methacarn-fixed samples of invasive breast carcinoma (Delfour et al., 2006).

In addition to staining intensity, the type of fixation may also influence the subcellular localization of the studied antigens. Discrepancies with the expected cytoplasmic localization of TGFβ1, FGF2 and IGF2 were demonstrated in rabbit chondrocytes when alcohol fixatives (Carnoy, methacarn) were used. These antigens showed predominantly nuclear positivity in the case of alcohol fixation, compared to formalin fixation (Bos et al., 2000). In contrast, better subcellular localization of desmocollin-1 in liver tissue after fixation with alcohol fixative (NEOFIX) was demonstrated compared to formalin. Formalin-fixed tissue showed cytoplasmic and weak membrane positivity, whereas NEO-FIX fixation resulted in a distinct membrane localization of protein (Paavilainen et al., 2010).

The negative influence on the intensity of IHC positivity caused by the duration of tissue slides archiving was described by a number of authors. Bertheau et al. reported a decline in IHC positivity in the detection of various nuclear, cytoplasmic and membrane localized antigens, as early as after three months of tissue slides archiving (Bertheau et al., 1998). Mirlacher et al. compared the IHC positivity of estrogen and progesterone receptors, cyclin D1, HER2, and E-cadherin in breast cancer patients (Mirlacher et al., 2004). These authors recorded a statistically significant decrease in IHC positivity for all the observed antigens, except for D1 cyclin, in tissue sections archived for six months, versus freshly prepared slides. Vis et al. studied the influence of the period of time tissue slides were archived on the immunopositivity of p27kip1, CD-44s, MIB-1, and androgen receptors in prostate tissue and they noted an exponential and significant decrease in IHC positivity for p27kip1, CD-44s, MIB-1, and AR, with half-lives of 587 days, 214 days, and 290 days for p27kip1, MIB-1, and AR, respectively (Vis et al., 2000).

The decline in IHC positivity in tissue blocks, dependent on the duration of archiving, was also demonstrated in the detection of various antigens in tissue blocks archived for various lengths of time. Previous studies (Blows et al., 2016; Dwork et al., 1998; Grillo et al., 2017; Litlekalsoy et al., 2007; Manne et al., 1997) dealing with the decrease of IHC positivity associated with the duration of archiving produced inconclusive results. However, it appears that different antigens vary in their sensitivity to the loss of antigenicity. Dwork et al. (1998), as one of the first, investigated IHC positivity of Alz-50 (paired helical filaments, ubiquitin and β-amyloid) in tissue blocks in patients with Alzheimer's disease, which were archived for 50 years. For Alz-50, IHC samples were positive after 10 years of archiving, but completely negative in blocks archived for 30 years. Conversely, Manne et al. did not demonstrate a decrease in the IHC positivity of p53 and Bcl-2 in colorectal carcinoma tissue blocks that had been archived for 3–16 years (Manne et al., 1997). These authors also note that the decline in IHC positivity in tissue blocks is relatively slow, and more a noticeable loss of positivity occurs after more than twenty years of archiving.

The influence of fixation and long-term archiving on tissue blocks of human embryo and fetal organs has not yet been described in literature. The aim of this study was to describe the influence of these factors on very rare experimental materials.

2. Materials and methods

2.1. Tissue samples

Tissue samples were obtained from the archive of the Department of Histology and Embryology, Faculty of Medicine and Dentistry, Palacky University in Olomouc. It contains tissue blocks from 1964 to 2008. It is a collection of 10% neutral buffered formalin- or methacarn-fixed, paraffin embedded tissue samples from clinically aborted embryos/fetuses in various gestation ages. All samples were presumed to be normal as no tissues were provided from cases known to have defects or adverse exposures. The tissue blocks were stored in the dark in metal drawer storage cabinets at room temperature over time.

In total, we used 12 samples of human embryonic/fetal tissue from three different organs: placenta (n = 4), intestines (n = 4) and liver (n = 4). We selected the tissue samples according to these criteria: formalin-fixed samples stored shorter than 20 years, formalin-fixed samples stored longer than 20 years and the same for methacarn-fixed tissue samples (in total, four samples from one organ). These criteria were followed for placenta, intestines and liver, respectively. For samples characteristics see Table 1. Unfortunately, no tissue samples that were stored for less than ten years or fresh tissue samples were available due to the general scarcity of this material. The use of the samples has been approved by the Ethics Committee of the University Hospital and the Faculty of Medicine and Dentistry, Palacky University in Olomouc.

2.2. Immunohistochemistry

We detected three different antigens with different subcellular localization: Ki-67, cyclin A and β-actin. The proteins of interests were detected in 4 μm thick paraffin sections using the indirect two step immunohistochemical method. These antigens were chosen for their expected high abundance in tissues. Ki-67 is a proliferation marker independent of the cell cycle phases and cyclin A is an important player in the regulation of the cell cycle: both of these proteins are commonly present in developing tissues. β-Actin is ubiquitously expressed in non-muscle tissue. The characteristics of used primary antibodies and their dilutions are summarized in Table 2. Appropriate dilutions of primary antibodies for immunostaining were determined by the staining of positive control samples as recommended by the manufacturer. The antibodies were diluted in Dako REAL™ Antibody Diluent (Dako).

Before immunostaining, the slides were deparaffinised and hydrated. To unmask the antigen, heat-induced antigen retrieval (HIAR) in citrate buffer pH 6 was performed. Moreover, methacarn-fixed tissues were also immunostained without HIAR for comparison. In order to block non-specific background staining, samples were incubated with Protein Block (Dako) for 30 min at room temperature. All samples were then incubated with primary antibodies for one hour at room temperature.

The detection of Ki-67, cyclin A and β-actin was performed by EnVision™ Detection System, Peroxidase/DAB, Rabbit/Mouse (Dako). Tris buffer (pH 7,6) was used for washing between the various steps. Nuclei of all samples were counterstained with haematoxylin. The samples were then dehydrated and coverslipped.

As a negative control, the primary antibody was substituted by Tris buffer, followed by incubation with the detection system. Positive and negative controls were present in the immunostaining of samples in order to verify the staining process.

Table 1
Characterization of used tissue samples.

No.	Tissue	Gestation age (weeks of IUD)	Fixation	Storage (years)
#1	Placenta	13	Formalin	12
#2	Intestines (small, proximal)	11	Formalin	17
#3	Liver	14	Formalin	12
#4	Placenta	12	Methacarn	15
#5	Intestines (small, proximal)	12	Methacarn	18
#6	Liver	12	Methacarn	15
#7	Placenta	7	Formalin	24
#8	Intestines (small, distal)	16	Formalin	45
#9	Liver	7	Formalin	24
#10	Placenta	7	Methacarn	22
#11	Intestines (small, proximal)	13	Methacarn	23
#12	Liver	12	Methacarn	23

Table 2
Characterization of used antibodies.

Antigen	Clone	Vendor	Souce	Clonality	Dilution	Expected localization
Ki-67	MIB-1	DAKO	Mouse	Monoclonal	1:200	Nucleus
cyclin A	H-432	Santa Cruz	Rabbit	Polyclonal	1:100	Nucleus, cytoplasm
β-Actin	C4	Santa Cruz	Mouse	Monoclonal	1:200	Cytoplasm, cell membrane

2.3. Evaluation of immunohistochemical staining intensities

Prior to evaluation, the samples were coded to minimize observer bias. The staining intensities were measured by ImageJ software and expressed as reciprocal staining intensities in graphs (reciprocal staining intensity = 250 − measured staining intensity) (Nguyen et al., 2013).

In each sample, the staining intensity of 25 cells in three different fields of vision (magnification 200×) was measured. To filter out possible different staining intensities in different cell types in tissues, we evaluated only certain types of cells. For Ki-67 and cyclin A, the nuclei of epithelial cells in intestinal crypts, nuclei of hepatocytes and nuclei of trophoblastic cells of placenta were measured. For β-actin, cytoplasmic/cell membrane positivity was measured in stromal cells of intestinal villi, stromal cells of placental villi and in endothelial cells of liver sinusoids. Individual reciprocal staining intensities are illustrated in the graphs, red horizontal line represents mean of reciprocal staining intensity ± SD.

2.4. Statistical analysis

The obtained staining intensities were evaluated by Mann–Whitney test to identify the differences between the used fixation method or the duration for which tissue samples were kept in storage at the level of significance $P < 0.05$. Statistically significant results are marked directly in the graph by asterisks (*). We use * for significant result ($P < 0.05$), ** for very significant result ($P < 0.01$) and *** and **** for extremely significant result ($P < 0.001$ and $P < 0.0001$ respectively). All calculations were performed with GraphPad Prism 6 software.

3. Results

3.1. Influence of fixation on immunohistochemical staining intensity

In case of methacarn-fixed tissues, the better staining results were obtained in samples which underwent HIAR. Because of this fact, the staining intensities were measured in samples stained with HIAR (see Supplementary files S2, S3 and S4 for comparison).

Regardless of the storage duration of tissue samples, if all three antigens were evaluated together, the measured staining intensities in formalin-fixed tissue samples were significantly higher than in methacarn-fixed tissue samples (n = 450; 150 cells for each of three antigens, 6 tissue blocks); Mann–Whitney test, $P < 0.0001$.

If the tested antigens were evaluated separately (n = 150; 25 measured cells from each of 6 formalin- or methacarn-fixed tissue blocks), nuclear antigens Ki-67 and cyclin A showed a statistically nonsignificant decrease in staining intensities with $P = 0.1355$ and $P = 0.0660$ respectively. Statistically very significant results were obtained for β-actin staining ($P = 0.0001$) as illustrated in Fig. 1.

3.2. Influence of duration of storage on immunohistochemical staining intensity

The tissue samples were divided into two groups according to the duration of storage. The tissues stored 10–19 years (6 blocks; mean 14.8 ± 2.5 years; median 15 years) and 20–45 years (6 blocks, mean 26.8 ± 8.9 years; median 23.5 years). The duration of storage differed significantly between these groups (Mann–Whitney test, $P = 0.0022$).

The duration of storage of tissue blocks affects staining intensities of Ki-67, cyclin A and β-actin. Representative microphotographs are shown in Fig. 2 (Ki-67) and Fig. 3 (β-actin). Storage of more than 20 years led to statistically significant fading of staining intensities. This effect was obvious regardless of the used fixation method. If the results were evaluated without regard for the fixation method, they showed extremely significant results with $P < 0.0001$ for all proteins of interest (n = 150; 25 measured cells from each of 6 blocks stored for 10–19 years and 20–45 years respectively; data not shown). When the staining intensities were evaluated separately in formalin- and methacarn-fixed tissue samples (n = 75 for each group: 25 epithelial cells of intestinal crypts, 25 hepatocytes, 25 trophoblastic cells of placenta), the trend was similar with P values in range from 0.0032 to <0.0001 (see Fig. 4). The evaluations of staining intensities separately for placenta, intestines and liver respectively are provided in Supplementary file S1. Corresponding microphotographs for immunostaining of antigens of interests are provided in Supplementary files S2 (Ki-67), S3 (cyclin A) and S4 (β-actin). The data indicated that the staining intensities decreased significantly in most cases (12 from 18) with longer storage of tissue blocks.

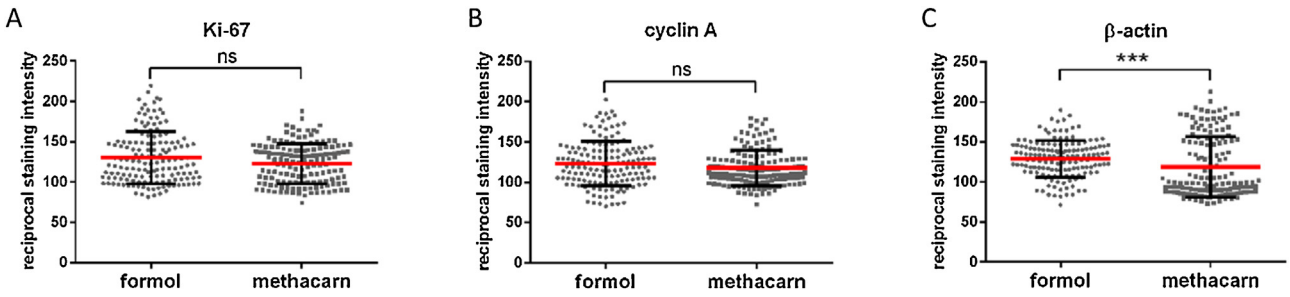

Fig. 1. Effect of tissue fixation on IHC staining intensity. Individual staining intensities for Ki-67 (A), cyclin A (B), β-actin (C). The data are expressed as mean (red line) ± SD. Each group represents measurement of 150 cells in total (50 epithelial cells of intestinal crypts, 50 hepatocytes, 50 trophoblastic cells of placenta). Mann–Whitney test, ns – non-significant result, * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

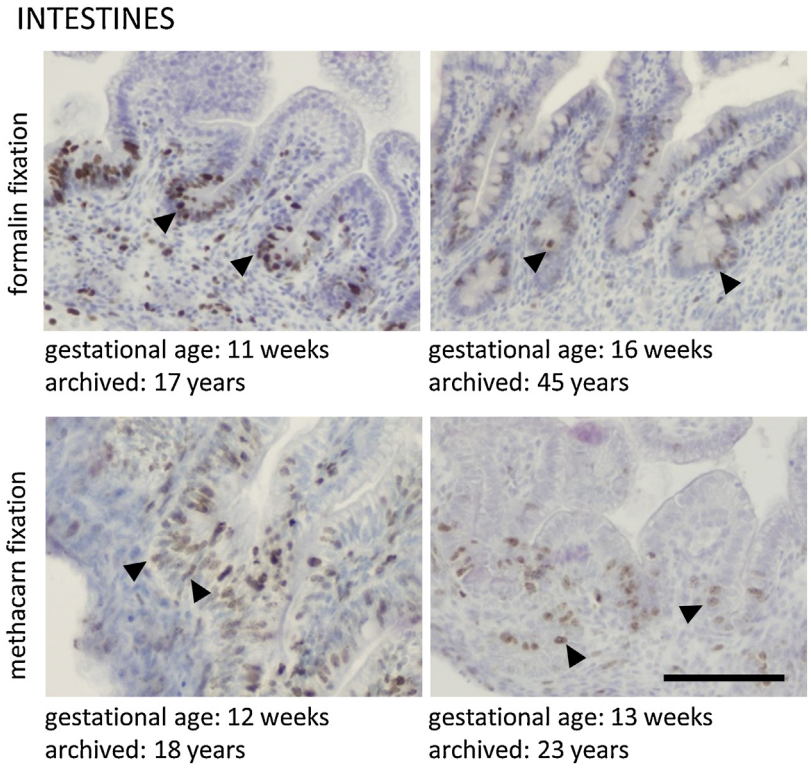

Fig. 2. Immunohistochemical staining of Ki-67 in crypt areas of intestines in formalin- and methacarn-fixed tissue samples. The gestation age and duration of storage is given directly under corresponding microphotograph. Note fading IHC signal in longer stored samples. Black arrow heads indicate examples of positive cells. All microphotographs are in same magnification (200×), the black line indicates distance of 100 μm.

4. Discussion

The aim of this work was to evaluate the effect which the used fixation type and the duration of tissue block archiving have on IHC intensity in human embryonic and fetal tissue material. In this study we have shown that fixation with 10% neutral buffered formalin achieved better IHC intensity results in comparison with methacarn-fixed tissues, and that the intensity of IHC staining declines with the length of time for which the tissue was archived.

It is known that the type of fixation affects IHC detection of various antigens, both the staining intensity and the subcellular localization. In general, fixation with formalin, or aldehyde fixatives generally, provides better morphological resolution and IHC staining results than other types of fixative (Bos et al., 2000; Dowsett et al., 2011; Mengel et al., 2002; Paavilainen et al., 2010), although there are exceptions (Delfour et al., 2006; Paavilainen et al., 2010; Warmington et al., 2000). It is in agreement with our results, when a decrease of IHC staining intensity was observed for all tested antigens in methacarn-fixed samples, although statistically significant difference was observed only for β-actin. Despite the observed decline of Ki-67 staining intensity, the difference was not statistically significant in our study. Other authors (Dowsett et al., 2011; Mengel et al., 2002) agree that in the case of this antigen, a fixation with agents other than formalin results in a reduction in dyeing. Although changes in subcellular localization of antigens after methacarn fixation were described (Bos et al., 2000), in our case, we did not observe differences in the subcellular localization of Ki-67, cyclin A and β-actin within human embryonic and fetal tissues fixed by formalin or methacarn.

The second aspect influencing IHC reactivity investigated in our study was the effect of the duration of tissue block archiving on IHC. Notwithstanding the other studies dealing with the influence of duration of storage on IHC results, to the best of our knowledge, this aspect has not as of yet been studied on human embryonic and

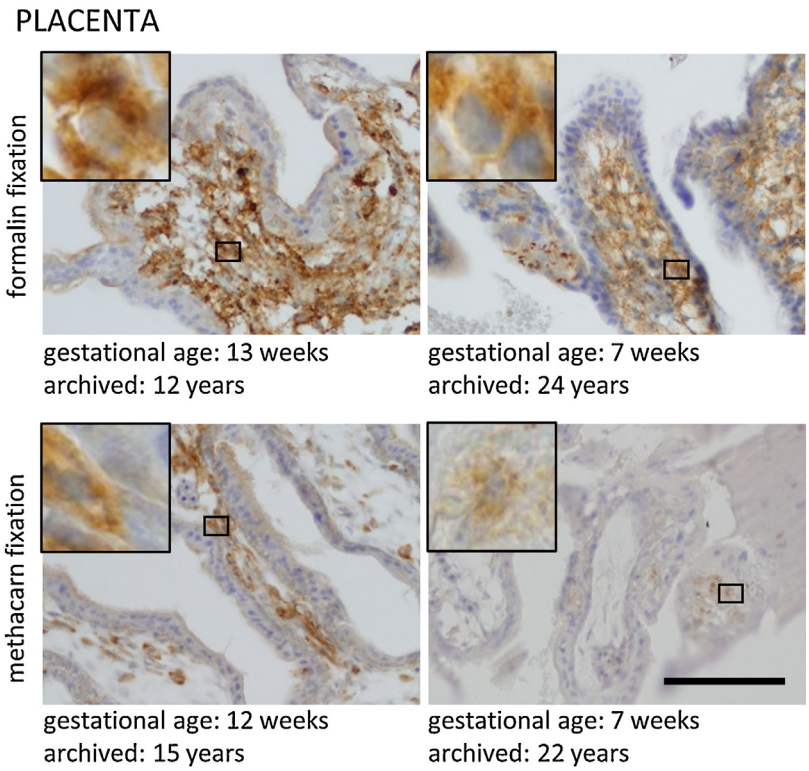

Fig. 3. Immunohistochemical staining of β-actin in placenta in formalin- and methacarn-fixed tissue samples. The gestation age and duration of storage is given directly under corresponding microphotograph. Note fading IHC signal in longer stored samples. Black arrow heads indicate examples of positive cells. All microphotographs are in same magnification (200×), the black line indicates distance of 100 μm. The examples of positive cells are given as insets in higher magnification (1000×).

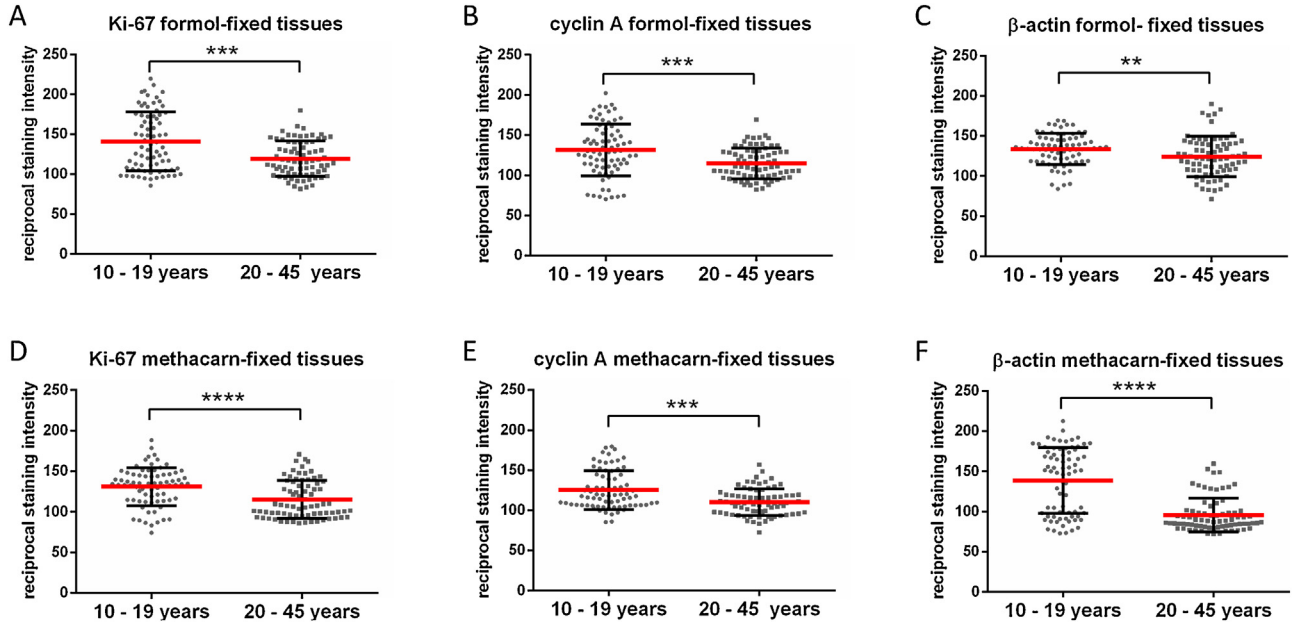

Fig. 4. Effect of duration of storage on staining intensity. Individual staining intensities for Ki-67 (A), cyclin A (B), β-actin (C) in formalin-fixed and methacarn-fixed (D, E, F respectively) tissue samples. The data are expressed as mean (red line) ± SD. Each group represents measurement of 75 cells in total (25 epithelial cells of intestinal crypts, 25 hepatocytes, 25 trophoblastic cells of placenta). Mann–Whitney test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fetal tissue blocks. In accordance with previously published studies (Blows et al., 2016; Dwork et al., 1998; Grillo et al., 2017; Litlekalsoy et al., 2007; Manne et al., 1997), we demonstrated a statistically significant decrease in IHC positivity for all studied antigens between samples that were archived for 10–19 and 20–45 years, in both formalin and methacarn formulations. In our study, the storage time for the most of "long term" stored samples was between 22 and 24 years. The only exception was a sample of formalin-fixed intestinal tissue stored for 45 years. We realized that 45 years of storage was a much longer period of time than for the others long stored tis-

sue samples we used. However, embryonic/fetal tissues are a rare material, and it was our only sample that met all criteria for tissue sample selection. The measured IHC staining intensities obtained for the 45 years old tissue sample do not differ from the five other used "long term" stored formalin-fixed tissue samples, which we believe, that the use of this particular block of tissue did not distort the results of our study.

In our study, the decrease in antigenicity was relevant to both the nuclear antigens Ki-67 and the cyclin A, as well as to the cytoplasmic β-actin. Recently, Grillo et al. (2017) formulated a hypothesis that subcellular antigen localization and the need for antigen retrieval has a negative influence on IHC positivity. These authors compared IHC intensity of a total of 12 antigens with different cellular localizations within formalin-fixed tissue block samples over the course of six decades: between 1960 and 2010. They demonstrated that nuclear and membrane antigens were more prone to loss of IHC positivity than antigens with cytoplasmic localization. These authors also demonstrated the highest loss of IHC positivity in the case of the Ki-67 nuclear antigen and also for the membranes localized antigen CD31 (Grillo et al., 2017). Our results support their findings for nuclear antigens, but differ in respect to cytoplasmic antigen β-actin. As well as Grillo et al., the decline in IHC positivity in cytoplasmic antigens was also reported by Litlekalsoy et al. (2007). They monitored IHC positivity of p53, p16, EGFR, cytokeratin 7 and HMW cytokeratin in tissue blocks from years 1932 - 2004. These authors reported a significant decrease in IHC positivity within blocks that had been archived for longer in the case of HMW cytokeratin but this was not the case for p53 and p16. Therefore, it appears that the type of antigen plays a more important role than the antigenś native subcellular localization on its own.

The causes of the decline in IHC positivity in archived tissue blocks are not well understood. According to some authors (Grillo et al., 2017; Xie et al., 2011), storage conditions may fundamentally influence the detection of antigens by IHC. These factors include, in particular, the ambient temperature, ambient humidity, air oxygen oxidation, and many others.

Our results could be relevant for clinical practice, where pathological archives represent a rich source of clinically identified tissue samples with great potential for retrospective studies. A possible decrease in IHC positivity over time should be borne in mind, particularly in this type of studies. It has been shown that Ki-67 labeling index is an independent risk factor influencing disease-free survival and overall survival in triplenegative breast cancer patients (Wang et al., 2016). These authors also demonstrated that patients with a high Ki-67 labeling index benefit better from carboplatin-based chemotherapy and that therefore, IHC Ki-67 positivity has a predictive as well as prognostic significance. Tashima et al. (2015) found that the cut-off point of 20% in the case of the Ki-67 labelling index is a prognostic factor for luminal HER-2 negative breast carcinoma. As mentioned above, our results and results obtained by Grillo et al. demonstrated that Ki-67 positivity decreases in tissue blocks with longer duration of storage (Grillo et al., 2017). This loss of antigenicity in tissue samples that have been archived for a longer period of time may distort the determination of the cut-off point.

5. Conclusion

In conclusion, we demonstrated that in archived human embryo and fetal tissue samples the fixation used and the duration of archiving have a significant effect on IHC positivity. To summarize our findings, better results were achieved in all formalin-fixed samples, while methacarn-fixed tissues showed a significant decrease in IHC positivity for β-actin. Furthermore, blocks of tissue archived for over 20 years showed significantly weaker IHC positivity for all studied antigens. Tissue blocks archiving for a period of time exceeding 20 years appears to be a critical period for the observed decline in IHC positivity.

Ethical statement

1) This material has not been published in whole or in part elsewhere;
2) The manuscript is not currently being considered for publication in another journal;
3) All authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aanat.2019.03.013.

References

Bertheau, P., Cazals-Hatem, D., Meignin, V., de Roquancourt, A., Verola, O., Lesourd, A., Sene, C., Brocheriou, C., Janin, A., 1998. Variability of immunohistochemical reactivity on stored paraffin slides. J. Clin. Pathol. 51, 370–374.

Blows, F.M., Ali, H.R., Dawson, S.J., Le Quesne, J., Provenzano, E., Caldas, C., Pharoah, P.D., 2016. Decline in antigenicity of tumor markers by storage time using pathology sections cut from tissue microarrays. Appl. Immunohistochem. Mol. Morphol. 24, 221–226.

Bos, P.K., van Osch, G.J., van der Kwast, T., Verwoerd-Verhoef, H.L., Verhaar, J.A., 2000. Fixation-dependent immunolocalization shift and immunoreactivity of intracellular growth factors in cartilage. Histochem. J. 32, 391–396.

Delfour, C., Roger, P., Bret, C., Berthe, M.L., Rochaix, P., Kalfa, N., Raynaud, P., Bibeau, F., Maudelonde, T., Boulle, N., 2006. RCL2, a new fixative, preserves morphology and nucleic acid integrity in paraffin-embedded breast carcinoma and microdissected breast tumor cells. J. Mol. Diagn. 8, 157–169.

Dowsett, M., Nielsen, T.O., A'Hern, R., Bartlett, J., Coombes, R.C., Cuzick, J., Ellis, M., Henry, N.L., Hugh, J.C., Lively, T., McShane, L., Paik, S., Penault-Llorca, F., Prudkin, L., Regan, M., Salter, J., Sotiriou, C., Smith, I.E., Viale, G., Zujewski, J.A., Hayes, D.F., 2011. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in breast cancer working group. J. Natl. Cancer Inst. 103, 1656–1664.

Dwork, A.J., Liu, D., Kaufman, M.A., Prohovnik, I., 1998. Archival, formalin-fixed tissue: its use in the study of Alzheimer's type changes. Clin. Neuropathol. 17, 45–49.

Grillo, F., Bruzzone, M., Pigozzi, S., Prosapio, S., Migliora, P., Fiocca, R., Mastracci, L., 2017. Immunohistochemistry on old archival paraffin blocks: is there an expiry date? J. Clin. Pathol. 70, 988–993.

Litlekalsoy, J., Vatne, V., Hostmark, J.G., Laerum, O.D., 2007. Immunohistochemical markers in urinary bladder carcinomas from paraffin-embedded archival tissue after storage for 5–70 years. BJU Int. 99, 1013–1019.

Manne, U., Myers, R.B., Moron, C., Poczatek, R.B., Dillard, S., Weiss, H., Brown, D., Srivastava, S., Grizzle, W.E., 1997. Prognostic significance of Bcl-2 expression and p53 nuclear accumulation in colorectal adenocarcinoma. Int. J. Cancer 74, 346–358.

Mengel, M., von Wasielewski, R., Wiese, B., Rudiger, T., Muller-Hermelink, H.K., Kreipe, H., 2002. Inter-laboratory and inter-observer reproducibility of immunohistochemical assessment of the Ki-67 labelling index in a large multi-centre trial. J. Pathol. 198, 292–299.

Mirlacher, M., Kasper, M., Storz, M., Knecht, Y., Durmuller, U., Simon, R., Mihatsch, M.J., Sauter, G., 2004. Influence of slide aging on results of translational research studies using immunohistochemistry. Mod. Pathol. 17, 1414–1420.

Nguyen, D.H., Zhou, T., Shu, J., Mao, J.H., 2013. Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity. Cancer InCytes 2.

Paavilainen, L., Edvinsson, A., Asplund, A., Hober, S., Kampf, C., Ponten, F., Wester, K., 2010. The impact of tissue fixatives on morphology and antibody-

based protein profiling in tissues and cells. J. Histochem. Cytochem. 58, 237–246.
Tashima, R., Nishimura, R., Osako, T., Nishiyama, Y., Okumura, Y., Nakano, M., Fujisue, M., Toyozumi, Y., Arima, N., 2015. Evaluation of an optimal cut-off point for the Ki-67 index as a prognostic factor in primary breast Cancer: a retrospective study. PLoS One 10, e0119565.
Vis, A.N., Kranse, R., Nigg, A.L., van der Kwast, T.H., 2000. Quantitative analysis of the decay of immunoreactivity in stored prostate needle biopsy sections. Am. J. Clin. Pathol. 113, 369–373.
Wang, W., Wu, J., Zhang, P., Fei, X., Zong, Y., Chen, X., Huang, O., He, J.R., Chen, W., Li, Y., Shen, K., Zhu, L., 2016. Prognostic and predictive value of Ki-67 in triple-negative breast cancer. Oncotarget 7, 31079–31087.
Warmington, A.R., Wilkinson, J.M., Riley, C.B., 2000. Evaluation of ethanol-based fixatives as a substitute for Formalin in diagnostic clinical laboratories. J. Histotechnol. 23, 299–308.
Xie, R., Chung, J.Y., Ylaya, K., Williams, R.L., Guerrero, N., Nakatsuka, N., Badie, C., Hewitt, S.M., 2011. Factors influencing the degradation of archival formalin-fixed paraffin-embedded tissue sections. J. Histochem. Cytochem. 59, 356–365.