FOLIA MEDICA CRACOVIENSIA
Vol. LVII, 4, 2017: 63–70
PL ISSN 0015-5616

Penetration of formaldehyde based fixatives into heart

Mateusz K. Hołda[1], Wiesława Klimek-Piotrowska[1], Mateusz Koziej[1], Kamil Tyrak[1], Jakub Hołda[1]

[1]HEART — Heart Embryology and Anatomy Research Team, Department of Anatomy, Jagiellonian University Medical College, Kraków, Poland

Corresponding author: Mateusz Hołda, PhD, MD
Department of Anatomy Jagiellonian University Medical College
ul. Kopernika 12, 31-034 Kraków, Poland
Phone/Fax: +48 12 422 95 11; E-mail: mkholda@gmail.com

Abstract: Objectives: To investigate the penetration depth of formaldehyde-based fixatives into cardiac muscle samples over the course of fixation.
Background: Fixation is the essential step in anatomical studies. However, very little is known about penetration of most common fixatives into cardiac tissue.
Methods: A total of 40 heart samples were investigated. 4 study groups (n=10 in each case) were formed in such manner they differed only in concentration and type of fixative (1) — 2% formaldehyde phosphate-buffered solution (FPBS); (2) — 4% FPBS (formalin); (3) — 10% FPBS; (4) — alcoholic formalin. Samples were measured before fixation and in the following time points: 24 hours, 72 hours, 168 hours.
Results: The penetration depth differed significantly among studied fixatives ($p<0.0001$). 100% penetration occurred in all samples after 72 hours in alcoholic formalin solution and after 168 hours in 10% FPBS. After alcoholic formalin fixation, the tissue is more brittle and sub-epicardial blisters were observed in some cases.
Conclusions: Alcoholic formalin solution is the fastest fixative among the studied ones, however it has several adverse effects on tissue structure. It was found that 10% FPBS is the best and a relatively fast fixative for cardiac morphometric studies.

Keywords: morphometry, formalin, alcoholic formalin, formaldehyde, heart anatomy, fixation.

Introduction

Chemical fixation is an essential step in anatomic, morphometric, and histopathologic examinations. Formaldehyde fixation is the most common method used for tissue fixation, and is a method of choice in both clinical and laboratory work [1]. Formaldehyde-based fixatives penetrates tissue well, but are relatively slow [2]. The mechanism of formaldehyde fixation is based on creating covalent chemical bonds between tissue proteins (crosslinking fixative) [3]. Formaldehyde penetration into the specimen is a physical process in which the solution diffuses and reaches the deepest cell layers [4]. This movement is governed by several physical factors and the sample size. Effective and fast fixation is limited to small samples (up to several millimeters thick). When using chemical fixation for anatomic purposes, scientists usually have to deal with a large, multiform tissue material that cannot be prepared in smaller pieces. Appropriate fixation of these samples is very difficult. Very little is known about the amount of time required to fix solid organs, including the heart.

Recent progress in invasive cardiac diagnostics and treatment procedures have triggered a wave of dozens of heart architecture morphometric studies per year, and these studies have been carried out on autopsied heart samples fixed in formaldehyde [5–8]which may help to plan CTI radio-frequency ablation. We examined 140 autopsied human hearts from Caucasian individuals of both sexes (29.3% females. Time is an important factor in chemical fixation, as it could influence the shape and size of the tissue that can be used [9]. However, very little is known about penetration of most common fixatives into cardiac tissue. Thus, the aim of this study was to investigate the penetration depth of formaldehyde-based fixatives into cardiac muscle samples over the course of fixation.

Materials and Methods

A total of 40 pig hearts were excised without delay after commercial animal (*Sus scrofa f. domestica*) slaughter, and were washed and stored in a low-temperature (8°C) saline solution until fixation time. No animal was deliberately sacrificed for this study, and all samples were originally intended for use in the food industry. The hearts were obtained intact. All hearts were cut in the same manner by the authors. The cut was performed on the right and left side of the interventricular septum. The right atrium was opened in a routine way using intercaval incision extending from the orifice of the superior vena cava to the orifice of the inferior vena cava.

The hearts were randomly assigned to one of four study groups (n = 10 each) that differed only in terms of their concentration and fixative type: (I) 2% formaldehyde

phosphate-buffered solution (FPBS); (II) 4% FPBS; (III) 10% FPBS; and (IV) alcoholic formalin solution (Alc; 85.5% absolute ethanol, 3.8% formaldehyde, 0.05% calcium acetate in distilled water). In no instance was the fixative-to-tissue ratio less than 40:1. All samples were stored in sealed containers at room temperature (21°C) between consecutive measurements.

The penetration depth of the fixing fluids was measured at the thickest site in the muscular tissue at 24 hours, 72 hours, and 168 hours of fixation time. During measurement, the hearts were removed from the fixative solution and dried at each of the three time points. The cut through the heart muscle was made in the thickest site of the sample and the distance from the epicardial surface to the visible border between the fixated and not-fixated tissue (i.e., distinct color changes or differences in tissue consistency) was assessed. Measurements were taken in 10 different points and the measurement mean was calculated, with measurements rounded to one decimal place. In total, three cuts were made in each heart. The cuts were always made in the thickest available point of the heart; the second cut (at 72 hour) was made at least 4 cm away from the first cut (at 24 hour) and the third cut (at 168 hour) was made at least 4cm from the other two. All measurements were taken using a 0.03 mm precision YATO (YT–7201) electronic caliper.

Statistical analysis was performed using the StatSoft Inc. software (STATISTICA v12, Tulsa, OK, USA). A p-value of less than 0.05 was taken to be statistically significant. Dataset distributions were verified to determine if there was normal distribution and homogeneity of the variances. The data was presented as mean values with 95% confidence intervals. Graphs were generated to illustrate changes. Comparisons within groups were made using repeated-measures analysis of variance (ANOVA) followed by Tukey's post-hoc test.

Results

The penetration depth differed significantly among the studied fixatives ($p < 0.0001$). Table 1 and Figure 1 present the mean (with confidence interval) penetration depth of different fixatives into the cardiac tissue over time.

In the first 24 hours of fixation, there was a statistically significant difference in penetration depth between 2% FPBS and other fixatives ($p < 0.05$) the penetration of which was lower. There were no statistically significant differences between 4%, 10% FPBS, and alcoholic formalin in the first 24 hours ($p > 0.4$). The 2% FPBS penetrated the tissue about 1.5 times slower.

After 72 hours, the difference in the penetration between the 2% FPBS and other fixatives persisted ($p < 0.04$), where 2% FPBS was still slower. There was no difference in penetration depth between 4% and 10% FPBS ($p = 0.0001$), however there was significant acceleration of the penetration speed observed in the alcoholic formalin

Table 1. Depth of penetration [mm] of different fixatives into the cardiac tissue among the time.

		24 hours					72 hours					168 hours				
Group	**N**	**mean**	**(-) CI95**	**CI95**	**min**	**max**	**mean**	**(-) CI95**	**CI95**	**min**	**max**	**mean**	**(-) CI95**	**CI95**	**min**	**max**
2% FPBS	10	4.5	3.7	5.4	3.3	7.4	7.1	6.1	8.2	4.8	8.0	10.8	9.1	12.5	7.6	13.1
4% FPBS	10	8.2	7.2	9.2	6.6	10.4	9.9	9.1	10.8	8.9	11.7	10.2	9.3	11.1	9.1	11.9
10% FPBS	10	7.0	6.4	7.6	6.2	7.8	11.4	10.2	12.6	10.7	13.6	17.7*	16.7	18.7	16.0	19.0
Alc	10	7.2	6.2	8.1	5.5	8.8	17.3*	15.7	18.9	15.0	19.5	18.9*	18.1	19.7	17.5	19.8

Alc — alcoholic formalin solution; CI — confidence interval; FPBS — formaldehyde phosphate-buffered solution; N — number of samples; * — 100% penetration in all samples

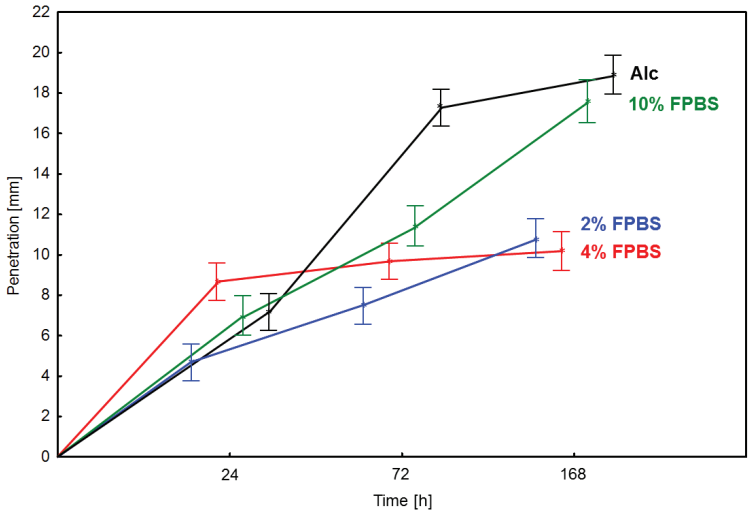

Fig. 1. Means and 95% coincidence intervals of tissue penetration depth [mm] into cardiac musculature in different fixative environments. Alc — alcoholic formalin solution, FPBS — formaldehyde phosphate-buffered solution.

solution compared with the other environments ($p = 0.0001$). Furthermore, 100% penetration of the alcoholic formalin solution was observed in all samples after 72 hours of fixation.

After one week of fixation, the samples stored in 2% and 4% FPBS were still not completely fixated. Differences in penetration depth between the 2% and the 4% FBPS became statistically insignificance ($p = 0.9$). However, 100% penetration of 10% FPBS was observed in all samples after 168 hours of fixation (Fig. 2).

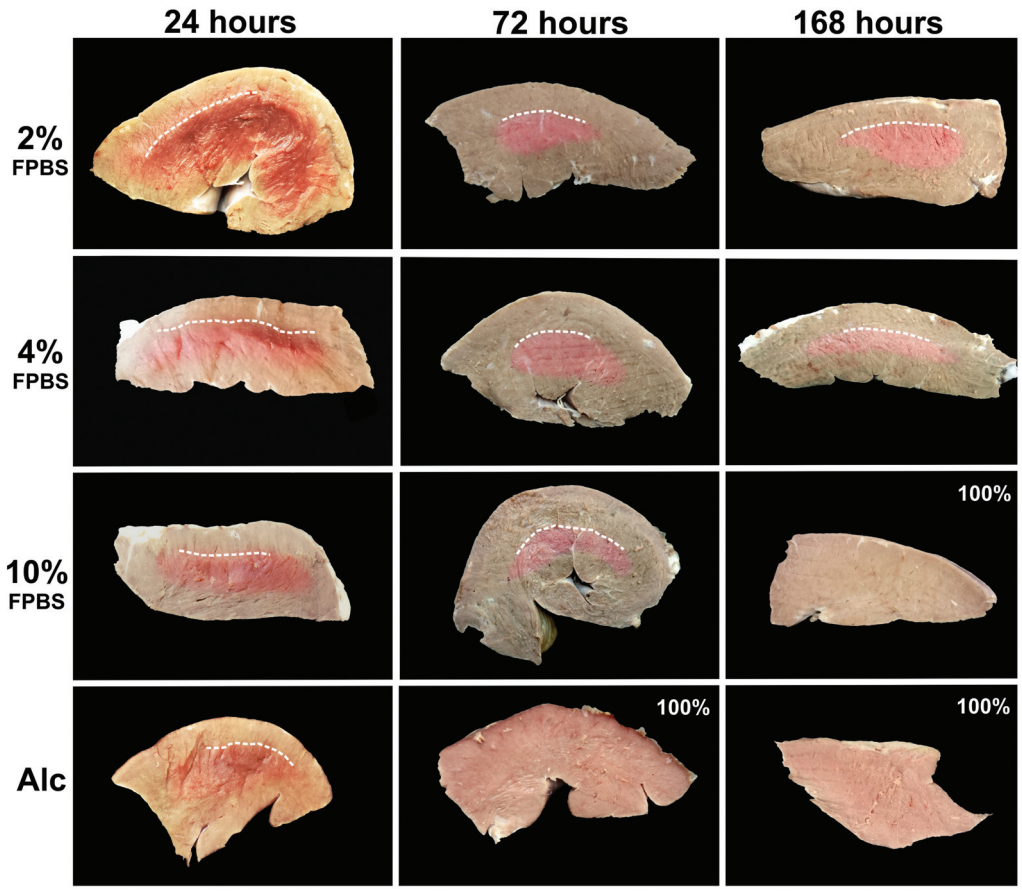

Fig. 2. Photographs of cadaveric heart specimens. The penetration depth of different fixatives into the heart ventricle could be observed. Alc — alcoholic formalin solution; FPBS — formaldehyde phosphate-buffered solution.

The muscular tissue color depended on the fixative type: beige for FPBS and purple for the alcoholic formalin solution (Fig. 3). After fixation in alcoholic formalin, the tissue was very brittle and would break easily along the muscle fiber course. Subepicardial blisters that were filled with fluid could be observed in this study group.

Discussion

Measurements of fixative penetration depth provided empirical basis for the minimum amount of time needed for tissue fixation. Penetration depth was possible to determine easily without the aid of chemical tests, or the use of complex techniques, since distinct color changes and differences in tissue consistency were induced in the

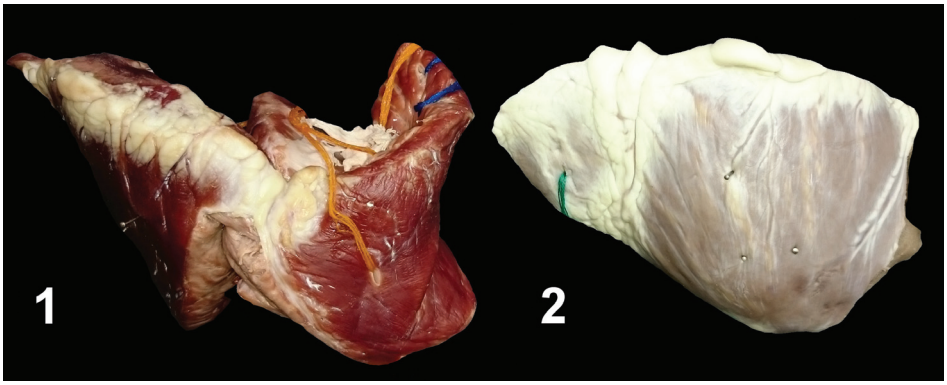

Fig. 3. Photographs of cadaveric heart specimens. Difference in the color of the muscular tissue was observed. 1 — alcoholic formalin solution; 2–10% formaldehyde phosphate-buffered solution.

penetrated regions [10]. The practical objective of this study was to provide the mean time for whole heart fixation and to identify the best fixative solution among the studied ones, as this information would be useful information for cardioanatomists.

The animal tissue used in this study did not differ from human tissue in terms of any anatomical aspects, therefore the results of the present study can be translated into human heart research in its entirety [11]. The penetration of formaldehyde-based fixatives in tissue is governed by Fick's law of diffusion. The penetration is dependent on the following: temperature, pH, solution volume, time, pressure, tissue type, tissue surface area, as well as fixative type and concentration [1]. In this study, all variables influencing the process (except fixative type and concentration) were identical in all study groups.

Traditional chemical fixatives can be divided into two main categories in terms of their action on proteins: coagulants (alcohols) and non-coagulants (aldehydes). Alcoholic formalin is a two-phase fixative, with initial alcohol fixation phase, followed by a cross-linking phase mediated by formaldehyde. In addition to fixation, dehydration and hardening of the tissue is observed as a result of the ethanol action. The use of alcohol as a formaldehyde diluent results in faster solution penetration into tissue, however tissue deformation may be observed [12]. Similarly, the results of this study demonstrated that the fastest penetration was observed in case of alcoholic formalin, yet the adverse effects on tissue structure disqualify this substance from being the ideal fixative.

Anatomical researches require fixing entire organs, yet cutting tissue into smaller parts that are easy to preserve is impossible due to necessity to assess complete material. As shown in this study, even after one week of fixation in 2% and 4% FPBS, only half of the tissue thickness was preserved in all samples, hence appropriate choice of suitable fixative and fixation time is crucial. This study confirms that concentrated

formaldehyde consistently penetrated more rapidly than less concentrated solutions [10]. Delayed fixation of tissue sites distant from the sample surface could influence observation results, thus larger specimens should be perfused to facilitate rapid and even fixation [13].

The 10% FPBS solution appeared to be the best fixative for cardiac morphometric purposes among those studied. Prior studies have shown that this solution does not cause any statistically significant changes in majority of the cardiac tissue parameters, whereas the other fixatives may have these issues [9]. In this study the 10% FPBS was also a faster fixative than 2% and 4% FPBS, which makes it a preferable reagent to use for cardioanatomists.

Conclusion

The penetration rate of a fixative is determined by the percentage concentration of formaldehyde and the solvent (phosphate-buffer or ethanol). The alcoholic formalin solution was the fastest fixative among the studied ones; however, it produced several adverse effects on tissue structure. Thus, 10% FBPS is the best fixative for cardiac morphometric studies, as it is relatively fast yet produces minimal effects on tissue structure.

Conflict of interest

None declared.

References

1. *Thavarajah R., Mudimbaimannar V.K., Elizabeth J., Rao U.K., Ranganathan K.*: Chemical and physical basics of routine formaldehyde fixation. J Oral Maxillofac Pathol. 2012; 16 (3): 400–405.
2. *Medawar P.B.*: The rate of penetration of fixatives. J R Microsc Soc. 1941; 61: 46–57.
3. *Sompuram S.R., Vani K., Messana E., Bogen S.A.*: A molecular mechanism of formalin fixation and antigen retrieval. Am J Clin Pathol. 2004; 121 (2): 190–199.
4. *Grizzle W.E., Fredenburgh J.L., Myers R.B.*: Fixation of tissues. In: J.D. Bancroft, M. Gamble (eds.), Theory and Practice of Histological Techniques. Philadelphia: Elsevier Limited, 2008; 56–63.
5. *Klimek-Piotrowska W., Hołda M.K., Piątek K., Koziej M., Hołda J.*: Normal distal pulmonary vein anatomy. Peer J. 2016; 4: e1579.
6. *Klimek-Piotrowska W., Hołda M.K., Koziej M., Hołda J., Piątek K., Tyrak K., Bolechała F.*: Clinical Anatomy of the Cavotricuspid Isthmus and Terminal Crest. PLoS One. 2016; 11 (9): e0163383.
7. *Klimek-Piotrowska W., Hołda M.K., Koziej M., Strona M.*: Anatomical barriers in the right atrium to the coronary sinus cannulation. Peer J. 2016; 1.
8. *Cabrera J.A., Pizarro G., Sánchez-Quintana D.*: Muscular architecture of the mitral isthmus: anatomical determinants for catheter ablation. Europace. 2012; 14 (8): 1069–1071.
9. *Hołda M., Klimek-Piotrowska W., Koziej M., Piątek K., Hołda J.*: Influence of different fixation protocols on the preservation and dimensions of cardiac tissue. J Anat. 2016; 229 (2): 334–340.

10. *Dempster W.T.*: Rates of penetration of fixing fluids. Am J Anat. 1960; 107: 59–72.
11. *Crick S.J., Sheppard M.N., Ho S.Y., Gebstein L., Anderson R.H.*: Anatomy of the pig heart: comparisons with normal human cardiac structure. J Anat. 1998; 193 (1): 105–119.
12. *Harrison P.T.*: An ethanol-acetic acid-formol saline fixative for routine use with special application to the fixation of non-perfused rat lung. Lab Anim. 1984; 18 (4): 325–331.
13. *Start R.D., Layton C.M., Cross S.S., Smith J.H.*: Reassessment of the rate of fixative diffusion. J Clin Pathol. 1992; 45 (12): 1120–1121.