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Research Article

Effects of Different Fixative Solutions on Kidney Volume [1] [2]

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Abstract

With this study, it is aimed to determine the volumetric changes at kidneys of 40 sheep (left and right kidneys, in total 80 kidneys) before and after treated with different fixation solutions by using a stereological method. In the study, 80 sheep kidneys provided from our faculty slaughterhouse were used. Kidneys were divided into four groups of 20 kidneys in each group (10 left and 10 right kidneys). During slaughtering of the sheeps, kidneys were rapidly taken out from abdominal cavity of the animal and fresh kidneys volumes which sectioned to 0.5 cm thickness were determined by Cavalieri Principle. It was noticed that this process did not exceed 30 min in order to prevent autolysis. Afterwards, groups of kidneys each including 20 were put into Formalin, Ethyl alcohol, Zenker and Bouin solutions and fixed upon determinated fixing durations. Volume redeterminations by using Cavalier Method were also performed on fixed kidney cross sections. Number of intersected points and other parameters were entered to the macro program (Microsoft Excel XP) and volumes were determined. Volumetric changes occured on kidneys before and after fixation were evaluated, and statistical calculations were performed. It was observed that all of the fixing solutions used in this study have different rate of diminish effect on kidney volume. However this effect were not statistically significant. The evaluation of interaction on volume changes; solution, direction and solution x direction were also not significant (P>0.05). We think that data gathered at anatomical and histological research using fixed materials may be used as references.

Keywords: *Fixative solutions, Kidney, Stereology, Volume*

Farklı Tespit Solüsyonlarının Böbrek Hacmi Üzerine Etkileri

Özet

Bu çalışma ile tespit öncesi ve farklı tespit solüsyonları kullanılarak fikse edilmiş 40 adet koyunun (sağ böbrek ve sol böbrek olmak üzere toplam 80 böbrek) böbreğindeki hacimsel değişikliklerin stereolojik bir yöntem kullanılarak ortaya konması amaç edinilmiştir. Çalışmada fakültemiz mezbahasından temin edilen 80 adet koyun böbreği kullanılmıştır. Böbrekler 20'şer (10 sağ ve 10 sol böbrek) olmak üzere 4 gruba ayrılmıştır. Koyun kesimi sırasında böbrekler hayvanın karın boşluğundan hızlı bir şekilde çıkartılmış ve 0.5 cm kalınlığında kesitlere ayrılan fresh böbreklerin Cavalieri prensibi ile hacimleri hesaplanmıştır. Otolizden korumak için bu işlem süresinin 30 dakikayı geçmemesine dikkat edilmiştir. Ardından 20'şerli gruplar halinde böbrekler Formalin, Etil alkol, Zenker ve Bouin solüsyonlarının içerisine konarak belirlenmiş sürelerde tespit edilmesi sağlanmıştır. Tespit olmuş böbrek kesitlerinin üzerinde Cavalieri prensibine göre tekrar hacim hesaplamaları yapılmıştır. Kesişen nokta sayıları ve diğer parametreler programa girilmiş ve hacim değerleri ortaya çıkarılmıştır. Tespit öncesi ve tespit sonrası böbrek hacimlerinde meydana gelen değişiklikler değerlendirilmiş ve istatistiki hesaplamalar yapılmıştır. Çalışmada kullanılan tespit solüsyonlarının böbrek hacmi üzerinde belirli oranlarda küçülme yarattığı gözlenmekle birlikte bu solüsyonların küçülme üzerine etkisinin istatistiki olarak önem taşımadığı belirlenmiştir. Hacim değişimi üzerine interaksiyonlar değerlendirildiğinde ise solüsyon, yön ve solüsyon x yön'ün böbrek hacmi üzerinde etkisinin de önemsiz olduğu gözlenmiştir (P>0.05). Yapılacak olan anatomik ve histolojik çalışmalarda fikse edilmiş materyaller kullanıldığında bu çalışmadan çıkarılacak olan verilerin referans olarak kullanılabileceğini düşünmekteyiz.

Anahtar sözcükler: *Fiksatif solüsyonlar, Böbrek, Stereoloji, Hacim*

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INTRODUCTION

Preserving the cells as they are in the live body is called fixation. The tissues that were taken out of the body will start degrading and spoiling in a short period of time unless specials measures are taken. Therefore, the tissues or organ parts taken should be fixed before any degradation occurs in the cells.

The requirements for fixation include selection and accurate preparation of an appropriate fixative solution, use of the solution 10 times higher in volume than the tissue volume [1,2], strictly following the fixation schedule, and selecting an appropriate temperature [3].

The agents used for the fixation are chemical substances. These are called as fixatives. A decent fixative should have lethal, penentrant, and hardening effects in the tissue. However, undesired characteristics are dissolution of the tissue components, shrink of the tissue, changes in the protein structure, and destruction of the nucleic acids [4].

The most frequently used fixative solutions are aldehydes, mercurials, alcohols, and picric acids. In the aldehyde group, there are Formaldehyde and Glutheraldehyde. Fixation is achieved by establishing cross bridges with the proteins of the tissue. 10% Formaldehyde has been used for fixing animal cadavers in the anatomy laboratories and fixing the tissues in histology and pathology laboratories. Mercurials contain mercury chloride. It is one of the most functional salts used for tissue fixation. It is seldom used alone becuase it cause shrinkage reaction. Its solutions are known as B5 and Zenker. Alcohols show their effects by causing denaturation of the proteins by dehyrating the tissue. They fix the tissue slowly but they harden and shrink the tissue. Ethyl and Methyl alcohols are in this group. Picric acid presicpitates all proteins and forms pitrat molecules that are water-soluble. It fixes rapidly. It cause shrink of the cell that can be easily seen [3,4]. The most important picric acid solution is Bouin solution.

Volume, concentration, temperature and time interval are among the factors affecting the fixation. Fixing procedure should start within maximum 30 min after the fresh tissue sample was taken because autolysis start immediately after death and specialized organs such as brain, kidney are more severely and faster affected than those rich in elastic fibrils and collagen. To be fixed sufficiently within 12-18 h, most tissues require a 10-15 times higher volume of freshly prepared fixative solution [4].

Cavalieri principle, which is a stereological method has been used frequently in a number of studies to calculate the volume of different tissues and organs [5-10]. Stereological methods have been widely used in recent years for evaluating the organs as a whole in experimental medical studies [9] as well as for evalutions at cell level [6,11].

Volumetric changes of the entire kidney or in its a certain part can be helpful for evaluation of kidney development, pathology and anomalies. Therefore, many studies on kidney volume focused on this issue. Unlike from previous studies, fresh kidney volumes and volumes of kidneys treated with different fixatives were calculated in our study. The tissues will be fixed by each of four groups of fixatives classified based on their mode of actions. The tissue fixatives usually work by shrinking the tissue which is an undesired effect. In this study, it was aimed to study volumetric changes in left and right kidneys of 40 sheep (total of 80) as fresh or after fixing in different solutions by a steorological method.

MATERIAL and METHODS

In this study, left and right kidneys of 40 sheep, total of 80, that were obtained from the teaching slaughterhouse of our faculty were used. The kidneys were divided into 4 groups of 20 in each (10 left and 10 right). During the dressing of sheep carcasses in the slaughterhouse, the kidneys were removed from the abdominal cavity and weighted using a digital scale (±0.01 mg of precision). As the first step of Cavalieri principle, the kidneys were cross-sectioned [12]. An electrical pastrami slicer with adjustable thickness feature was used for cross-sectioning. Depending on the size of the kidneys, 9 to 12 slices were obtained from each kidney. Then, 0.5 cm thick slices were arranged as the surfaces of the same direction will be upward. For the whole kidney volume, a point grid with 0.5 cm interval (distance between the encircled points) was used. Point grid is a transparent paper with points spaced equally on it *(Fig. 1)*. The point grids were randomly tossed onto the crossections of the kidneys and the numbers of encircled points on the tissue were used for calculating the kidney volume *(Fig. 2)*.

The points on the cross sections were counted and inserted in the appropriate places in below equation. The equation was computerized with a macro program prepared using Microsoft Excel (XP) *(Fig. 3)*.

$V = t \times a/p \times \sum P$

V = Volume; t = Section thickness; a/p = Area represented by each point on point grid; ∑P = Total point count on section surface area

This procedure was repeated for each cross section and finally the volume of the whole kidney was calculated. The macro program also automatically calculated the volume and error (CE).

Each of the fresh kidney section was then placed into the fixative solution in a locked jar by keeping the same direction of the surfaces used for measurements. The jars were groupped as to contain 10 left and 10 right kidneys for each fixative solution. The fixative solutions included

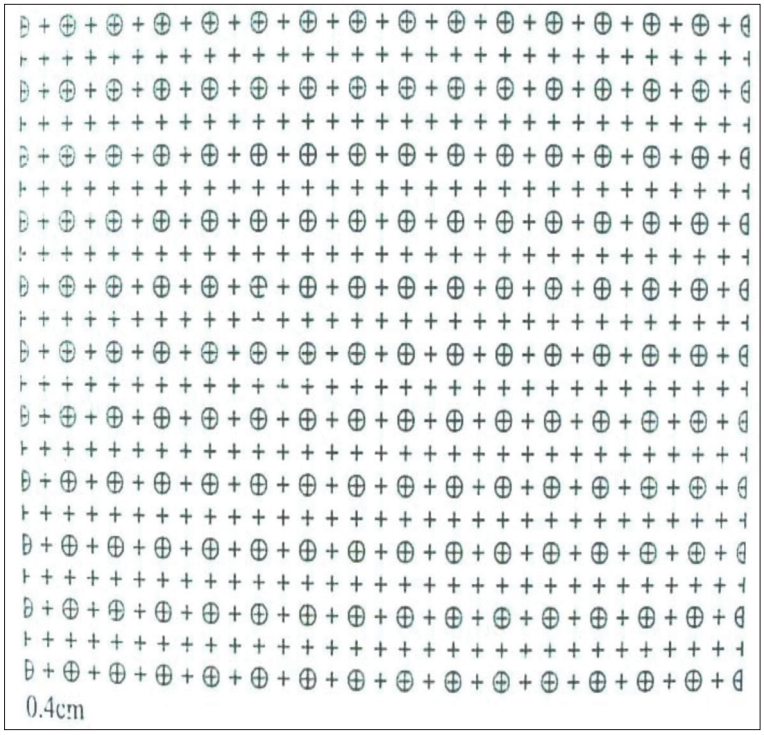
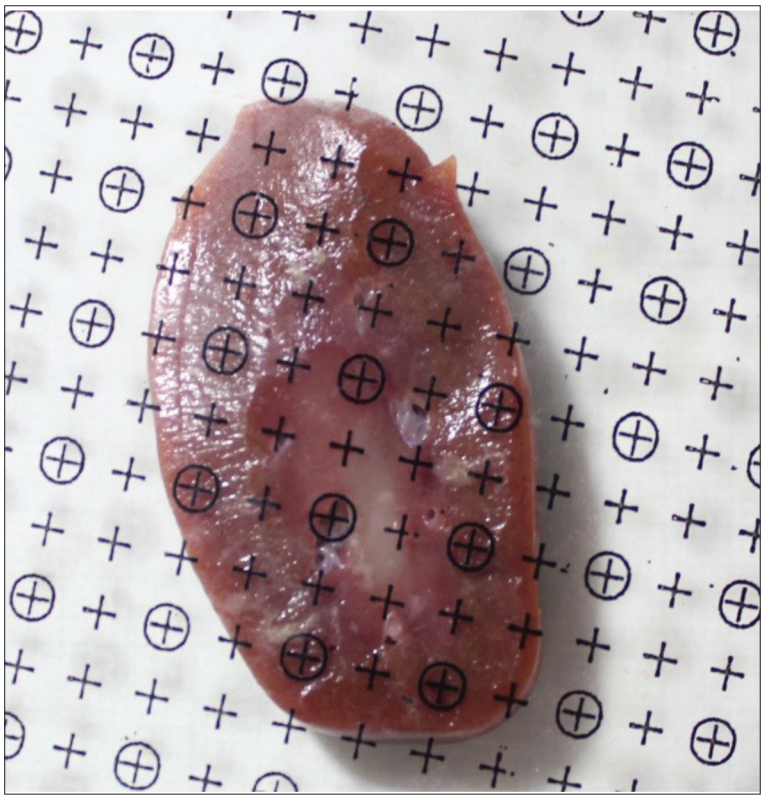


Fig 1. Point grid

Şekil 1. Nokta sayım cetveli

Fig 2. Superimposition of the point grid onto the crossections of the kidneys

Şekil 2. Böbrek kesitleri üzerine rastgele atılan bileşik noktalı alan ölçüm cetveli



Formalin, Ethyl alcohol, Zenker, and Bouin. The commercial 37% Formaldehyde was diluted with distilled water to 10% Formalin. Ethyl alcohol was prepared by mixing absolute alcohol and formalin. Zenker's fixative solution was prepared by using $K_2Cr_2O_7$, $HgCl_2$, glacial asetic acid and distilled water as described in the literature. Bouin solution was made from picric acid, formalin, and glacial acetic acid [2,3].

Fixation times of the numbered sections of the 10 left and 10 right kidneys were 24 h for Formalin, 24 h for Ethyl alcohol, 12 h for Zenker solution, and 18 h for Bouin solution. The amount of the fixative solutions in the jars were at least 10 fold of the volumes of the tissues. At the end of the fixation procedure, the tissues were weighted (±0.01 mg of precision) and all step that were used for the

Fresh:	Whole kidney
Solution:	
Direction	Counted
Section number	Point
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	
13	
14	
15	
16	
17	
18	
19	
20	
Total number of points hitting the sectior	0
Number of section=	
(t) Section thickness=	0,5
(SL) Scala lenght (measured by ruler)=	1
(SU) Scala unit=	1
(d) Distance between the points of the grid	0,5
b/a	10
	0,50
A	0,25
Nug	0,00
Variance of total area (Var srs)	0,00
Total variance	0,00
%nug	#SAYI/0!
CE (Coefficient error)	#SAYI/0!
Kidney Volume	0,00
Weight	

Fig 3. A macro program prepared using Microsoft Excel (XP)

Şekil 3. Microsoft Excel (XP sürümü) programı kullanılarak hazırlanmış olan makro program

volume calculations of the fresh sections were repeated on the fixed sections. The numbers of the intersecting points on the surfaces and other parameters were inserted to the program and volumes were calculated.

The changes in the volumes and weights of the left and right kidneys before and after the fixing were evaluated and statistical analysis were carried out. GLM procedures of the SPSS 13.0 software were used to determine the effects of the type of solution and the direction on the ratio of changes in weight and volume. The fixed effects of solution, direction, and solution x direction interaction were included in the GLM model. The photographs were taken by using Canon 550d brand camera.

RESULTS

The effects of fixative solution, direction and their interaction were not significant (P>0.05). The reduction in the volumes after fixing were found as 5.21% in Bouin solution, 6.4% in Ethly alcohol, 4.83% in Formalin, and 5.44% in Zenker's solution.

Effect of the fixative type on the weight change were significant (P<0.001). There was 26.91% decrease in Ethyl alcohol solution. The difference between Ethyl alcohol and other fixatives were significant. A 2.7% increase in Zenker solution was determined but this increase was lower compared to those found in Bouin and Formalin solutions. The difference between Bouin solution and Formalin was not significant *(Table 1)*.

DISCUSSION

The swelling or shrink of the cells occurs as an adverse effect of the fixation depending on the type of solution. As long as the effects of the chemical agents called as fixative is known, there will be less chance for error. Ethyl alcohol diffuse to the tissue slowly causing hardening and shrinking. Mercury chlorid and picric acid always cause shrinkage while glacial acetic acid results in the swelling of the cells, thus it is not used alone [2,3]. According to Fox *et al.*[13], the most important effect of Formaldehyde and other fixatives is to cause shrinkage. Many researchers attempt to explain how such shrinkage occur. However, these may result in many discrepancies because such shrinkage phenomenon was not determined by observation but by taking measurements before and after the fixing procedure [14-16].

In a study on rabbit kidney by Bolat *et al.*[14], the researchers observed that fixing the kidneys with Formalin

Table 1. *The effect of solution, direction and solution-direction on volume and weight change*

Tablo 1. *Hacim ve ağırlık değişimi üzerine solüsyonun, yönün ve solüsyon-yön interaksiyonunun etkisi*

Property	Solution (S)				Direction (D)		SEM	Significance		
	Bouin	Ethyl alcohol	Formalin	Zenker	Right	Left		S	D	SxD
Volume change, %	-5.21	-6.40	-4.83	-5.44	-5.78	-5.16	0.311	NS	NS	NS
Weight change, %	10.19a	-26.91c	9.62a	2.70b	-1.17	-1.03	0.298	***	NS	NS

**** P<0.001;* ***NS:*** *P> 0.05;* $^{a,b,c:}$ *Mean values carried different letters in the same line significantly different (P<0.05)*

caused an increase in the volume of the kidneys. Similarly, Warui and King [16] reported an 18% increase in volume of chicken kidney after fixing with glutaraldehyde via perfusion method. In contrary, Malas *et al*.[15] reported that Formaldehyde resulted in a 48.16% shrinkage in rat kidney volume. In our study, the volume of sheep kidney reduced at 4.83% when it was fixed in Formalin. Volumes of fresh organs was determined using the fluid replacement principle (Archimedes principle) in all previous studies, in our study, we used Cavalieri method to carry out a reliable comparison of fixed and fresh kidney volumes.

CE prediction is essential in stereological studies for deciding whether the frequency of the points on the grid and the numbers of the sections were sufficient. In general, a CE value of less than 10% is acceptable [17]. Şahin *et al*.[10] reported, the upper limit for CE as 5% in their study. In our study, the average CE value calculated for each section using Microsoft Excel program was found as 5%. It is thought that this value would be even lower if the numbers of the sections increase. However, we should note that slicing the fresh kidney by the slicer was somewhat difficult because the tissue was too soft, thus increasing the numbers of sections was not possible.

The experimental studies revealed that the Formalin had cancerogenic effect as well as harmful effects on many other systems including nervous system [18] and reproductive system [19]. Serious physical symptoms, irritation of eyes, lacrimation, irritation of airways and dermatitis are among the adverse effects that students and academicians experience [20]. Despite all these harmful effects, the reasons why this fixatives is still commonly used in making cadaver includes the low cost, ease of use [21], and high toxicity on the microorganisms [22]. When effects of the fixatives on the kidney volume was evaluated, which was also the objective of the current study, the least shrinkage of 4.83% was observed in Formaldehyde-fixed kidneys. In the literature, to our knowledge, there is no published study on the effects of Zenker's or Bouin's fixative solutions on the volume of organs. The reasons why Zenker and Bouin solutions are preferred for fixing biopsy materials which are small in size are thought to be difficulty of preparation of the solutions, higher cost of the chemical substances used in the formula of the solutions. In addition, coloring tissues to yellow and rendering the tissues fragile are considered as disadvantages of Bouin solution.

It is concluded that the difference in weight after fixing the tissues results from different mechanisms of the fixatives. Bolat *et al*.[14] fixed kidney with Formaline and found the weight increase was 7.33% in the left and 7.56% in the right kidney. In our study, the weight increase after fixing with Formaldehyde was 9.62% on average. Increase in weight was also observed after fixing with Zenker and Bouin solutions while Ethyl alcohol resulted in loss of weight after fixing. It has been thought that this loss might be due to the dehydrating effect of ethyl alcohol or breaking down the lipids.

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