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Effect of routine pathological procedure on morphometric parameters of the heart in rat models

Ozgur Ozdemir*, Orhan Yavuz and Fatih Hatipoglu

Department of Pathology, Faculty of Veterinary Medicine, Selcuk University, Konya Campus 42079, TURKEY.

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In this study, the aim was to investigate effects of routine protocols commonly used in pathology (formalin fixation, paraffin embedding, slicing and staining) on morphometrical parameters of hearts in rats. Six males and 6 females rats were used. All of the subjects were euthanized and then hearts were fixed in formalin solution. Hearts were cut at the level of musculus papillaris and cut surfaces area were photographed (Formalin group). Thereafter, cut surfaces of the hearts embedded in paraffin were also photographed (Paraffin group). Staining was done using H&E staining on 5 µm slices and their photographs were taken (Slide Group). Analyses of thickness, diameter and area on the photographs of heart were done by using image analysis software. Left ventricular mass (LVM), left ventricular mass index (LVMI) and cut surface area (CSA) were calculated. Significant differences were found in LVM and LVMI values between Paraffin and Formalin groups ($p<0.05$). Comparisons between males and females in accordance to their respective groups showed that there were no differences in Slide group ($p>0.05$) but there were significant differences in CSA values of Paraffin and Formalin group ($p<0.05$). In conclusion, in case reports, in which left ventricular hypertrophy is investigated by the help of LVM and LVMI, Formalin and Slide group method and results might be more useful. On the other hand, all the three methods might be used in experimental studies included in healthy control groups. Additionally, it was discussed that the gender has no effect on evaluation among groups in terms of LVM and LVMI.

Key words: Morphometry, heart, rat, routine pathology, left ventricular mass (LVM)

INTRODUCTION

It is prominent to utilize echocardiographical examinations in investigations of heart abnormalities on humans and especially in determination of heart hypertrophy, the data obtained from echocardiography is analysed by certain calculations (Casiglia et al., 2008; Devereux and Reichek, 1977; Gosse et al., 1999; Krauser and Devereux, 2006; Urhausen et al., 2004). The used method in investigation of left ventricular hypertrophy with echocardiography was developed by Devereux and Reichek (1977) and utilized in postmortal examinations by using ruler and caliper (Fineschi et al., 2001, 2007; Montisci et al., 2012; Ozdemir et al., 2013). Again, it has been echocardiographically seen that postmortal morphometrical examinations on hearth were carried out in experimental animals but in some cases the examinations were done immediately following deaths

*Corressponding author. E-mail: oozdemir@selcuk.edu.tr

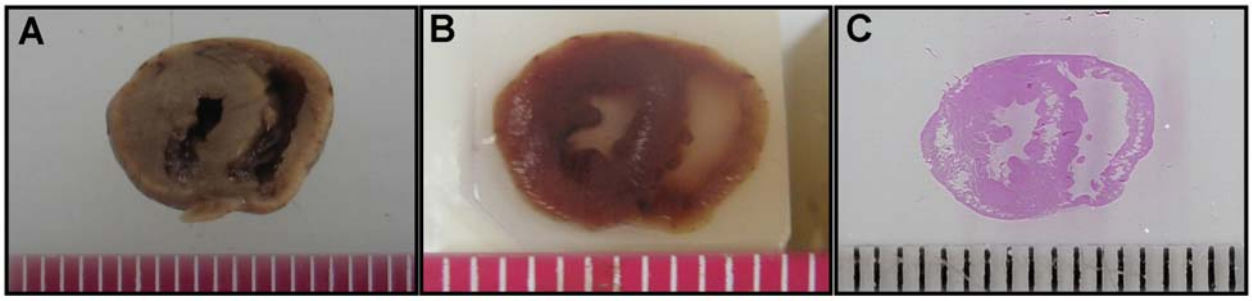


Figure 1. Sample of heart sections in routine histopathological procedure. (**A)** Formalin Group, (**B)** Paraffin Group, (**C)** Slide Group.

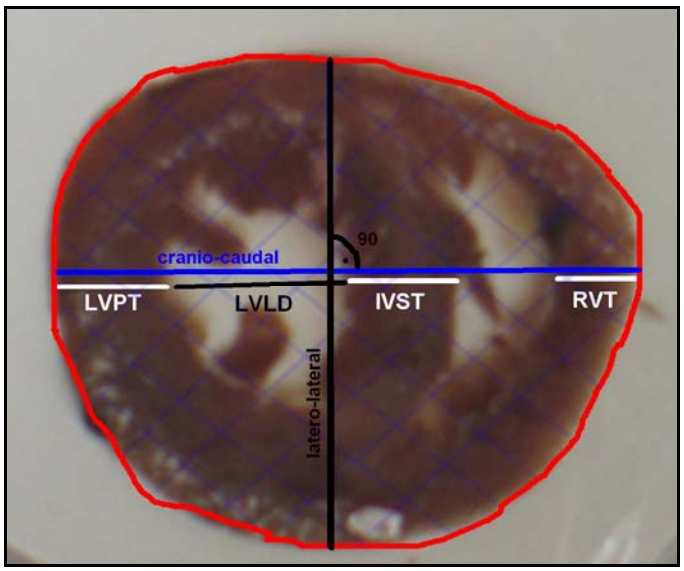


Figure 2. Measurement referance points on cut surface area of heart (Ozdemir et al., 2013). LVPT: Left ventricular posterior wall thickness; IVST: Interventricular septal thickness; LVLD: Left ventricular lumen diameter; RVT: Right ventricular thickness; CCD: Cranio-caudal diameter; LLD: Latero-lateral diameter; CSA: Cut surface area (scanned area).

and in some cases these examinations were done after section and staining are completed (Hinton et al., 2008; McAdamms et al., 2010; Noszczyk-Nowak et al., 2009; Woythaler et al., 1983). In retrospective studies, calculations were done in tissues usually after the formalin fixation (Chugh et al., 2000; Mannan et al., 2005).

Fixation, paraffin wax embedding and slicing are important processes of the routine pathology in postmortal investigations. Tissues usually somewhat shrink during formalin fixation and paraffin embedding. During the slicing process, the time spent between the sections wait in waterbath and transferring them on to lam, allows slices to expand and deteriorate (Luna, 1968; Unsaldi and Ciftci, 2010) and it could causes mistakes in morphometrical measurements.

In the present study, the aim was to investigate the effects of the routine fixation and tissue processing protocols commonly used in pathology (Formalin fixation, paraffin embedding, slicing and staining) on morphometrical parameters of the heart obtained from male and female rats.

MATERIALS AND METHODS

Six male and 6 female rats, 6 weeks of age, were used in the study. During the course of the study all rats were housed in polycarbonate cages (Techniplast, Italy) as 1 rat per 250 cm^2 and fed *adlibitum* with standart rat feed (Purina, Canada) and fresh water. All rats were euthanized under thiopental (40 mg/kg) anesthesia after body weight was measured. The hearts were wholly fixed in 10% formalin solution after heart weights was obtained. Then, they were transversally cut at the level of musculus papillaris and cross section surface were photographed (Formalin group) (Figure 1A). After routine tissue processing and parafin embedding, cut surfaces of paraffin wax tissues were photographed (Paraffin group) (Figure 1B). 5 µm thick sections were sliced at microtome and stained with Hematoxylin and Eosin (H&E). Also, stained preparations were photographed (Slide Group) (Figure 1C). Thickness, diameter and area of heart cut surfaces were analysed on photographs by using image analysis software (Digital Life Science Imaging, analySIS® LS Starter, 2.2, Build 1110, 23 An Olympus Company, Münster, Germany) in all groups. The values were measured as seen in Figure 2 (Ozdemir et al., 2013) in parameters of left ventriculus posterior thickness (LVPT), interventricular septum thickness (IVST), left ventriculus lumen diameter (LVLD), right ventriculus thickness (RVT), cut surface area (CSA), left ventricular area (LVA), right ventricular area (RVA), cut surface craniocaudal (CCD) and cut surface laterolateral (LLD) diameters (two measurement lines set right angle). Left ventricular mass (LVM) and left ventricular mass index (LVMI) were calculated according to Devereux and Reichek (1977), body surface area (BSA) was calculated according to Erer and Kiran (2005) after analyses.

$LVM\ (g) = 1.04\ ([LVPT + LVLD + IVST]^3 - [LVLD]^3) - 14\ g;$

$LVMI\ (g/m^2) = LVM / BSA$

$BSA(m^2) = [body\ weight\ (kg)]^{2/3} \times K/100$

The K value is a special factor to species and is accepted as 9 for

Table 1. Distribution of body weight, relative organ weight and body surface area values on groups of both male and female.

Analytical parameters	Male	Female
Body Weight (g)	271.00 ± 11.35[a]	221.50 ± 10.41[b]
Heart Weight (g)	1.06 ± 0.21[a]	0.87 ± 0.08[a]
Relative organ weight	0.39 ± 0.09[a]	0.39 ± 0.03[a]
Body surface area (m^2)	0.04± 0.00[a]	0.03 ± 0.00[b]

Different letters *(a, b, c)* in the same line indicate a statistically significant difference (p<0.05).

Table 2. Distrbution of length, area measurements and indexes of the heart on groups.

Analytical parameters	Male			Female		
	Formalin	Paraffin	Slide	Formalin	Paraffin	Slide
LVPT (mm)	3.51 ± 0.33[a]	2.56 ± 0.22[b]	3.05 ± 0.22[c]	3.37 ± 0.20[a]	2.52 ± 0.15[c]	2.93 ± 0.21[b]
IVST (mm)	2.67 ± 0.47[a]	2.05 ± 0.09[b]	2.24 ± 0.34[b]	2.67 ± 0.4334[a]	2.08 ± 0.40[b]	2.23 ± 0.27[b]
LVLD (mm)	2.54 ± 0.72[a]	2.74 ± 0.41[a]	2.28 ± 0.69[a]	2.19 ± 1.19[a]	2.65 ± 0.73[a]	2.49 ± 0.54[a]
RVT (mm)	1.49 ± 0.23[a]	1.07 ± 0.17[b]	1.20 ± 0.25[b]	1.64 ± 0.27[a]	1.10 ± 0.12[b]	1.34 ± 0.31[b]
CCD (mm)	11.37 ± 1.29[a]	9.59 ± 0.37[b]	10.50 ± 0.87[ab]	10.55 ± 0.31[a]	8.50 ± 0.46[b]	9.86 ± 0.65[a]
LLD (mm)	10.31 ± 1.16[a]	8.18 ± 0.62[b]	8.83 ± 0.60[b]	9.07 ± 0.16[a]	7.41 ± 0.22[c]	8.23 ± 0.41[b]
CSA (mm^2)	96.19 ± 14.65[a]	61.96 ± 5.55[c]	75.31 ± 8.07[b]	78.16 ± 3.98 [a]	50.60 ± 5.64 [c]	66.13 ± 8.27 [b]
LVA (mm^2)	3.69 ± 1.99[a]	3.45 ± 0.99[a]	3.24± 1.51[a]	1.97 ± 0.84 [a]	2.97 ± 1.16[a]	2.64 ± 0.78[a]
RVA (mm^2)	6.18 ± 3.93[a]	4.63 ± 2.67[a]	5.35 ± 4.12[a]	3.20 ± 0.23[a]	3.00 ± 1.33[a]	3.93 ± 1.78[a]
LVM (mg)	700.4 ± 314.1[a]	381.93 ± 61.48[b]	448.1 ± 98.6[ab]	563.0 ± 167.6[a]	364.93 ± 75.18[b]	442.5 ± 120.0[ab]
LVMI (g/m^2)	18.58 ± 8.44[a]	10.14 ± 1.70[b]	11.98 ± 5.49[ab]	17.07 ± 5.64[a]	11.11 ± 2.379[b]	13.5 ± 3.95 [ab]

Different letters (*a, b, c)* in the same line indicate a statistically significant difference (p<0.05). LVPT: Left ventricular posterior wall thickness, IVST: Interventricular septal thickness, LVLD: Left ventricular lumen diameter, RVT: Right ventricular thickness, CCD: Cranio-caudal diameter, LLD: Latero-lateral diameter, CSA: Cut surface area, LVA: left ventricular area, RVA: Right ventricular area, LVM: left ventriculus mass, LVMI: left ventriculus mass index, BSA: Body surface area.

mice and rats, 10 for cats, 10.1 for canines and 10.6 for a human of 70 kg body weight (Erer and Kiran, 2005).

Statistical analyses of the data were done using SPSS 13.0 pocket software (SPSS 13.0 for Windows/ SPSS® Inc, Chicago, USA). Comparison of the data between groups were done using ANOVA and Duncan tests, and between genders were done using independent t test. The values of p<0.05 was accepted as significant and results stated in Mean±SD.

RESULTS

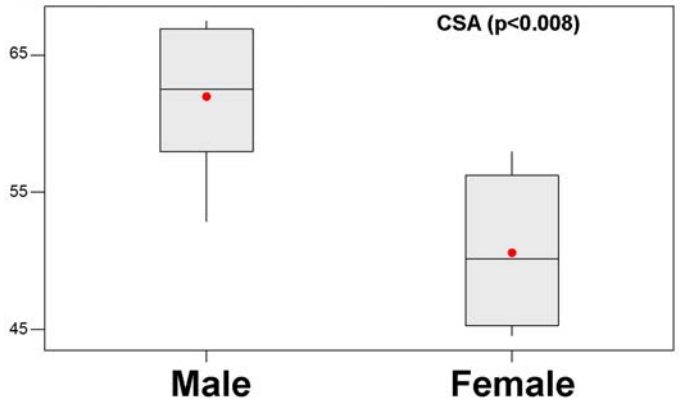
Distribution of live body weight, relative organ weight and body surface area values on male and female groups were shown in Table 1, and measurements and calculations of the heart were shown in Table 2.

As an overall examination of the data, the highest values with exception of LVLD were seen in the Formalin group whereas the lowest values were in the Paraffin group both in males and females. As for LVLD value, the highest was seen in Paraffin group and the lowest in slide group among males whereas the highest value was in Paraffin group and the lowest was in Formalin group among females. While significant differences were seen in LVPT and CSA values both in males and females among the groups (p<0.05), the differences between groups are insignificant in LVLD, LVA and RVA values (p>0.05). In point of IVST, the Paraffin and Slide groups were similar but Formalin group showed significant differences (p<0.05). The LVM and LVMI values had significant differences between Paraffin and Formalin group (p<0.05), but Slide group values located between the Paraffin and the Formalin groups and had no stastical difference.

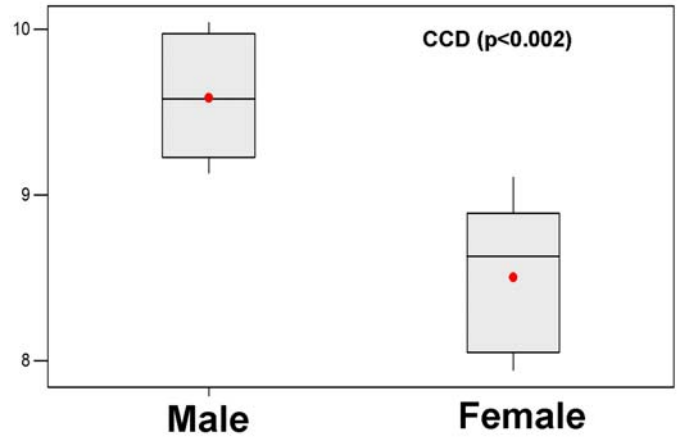
In comparisons of males and females according to the groups, there were no differences in Slide groups (p>0.05) whereas there were significcant differences in CSA (p<0.008), CCD (p<0.002) and LLD values (p<0.03) in Paraffin groups (Graphs 1 to 3) and there were differences in CSA values (p<0.05) (Graph 4) in Formalin groups.

DISCUSSION

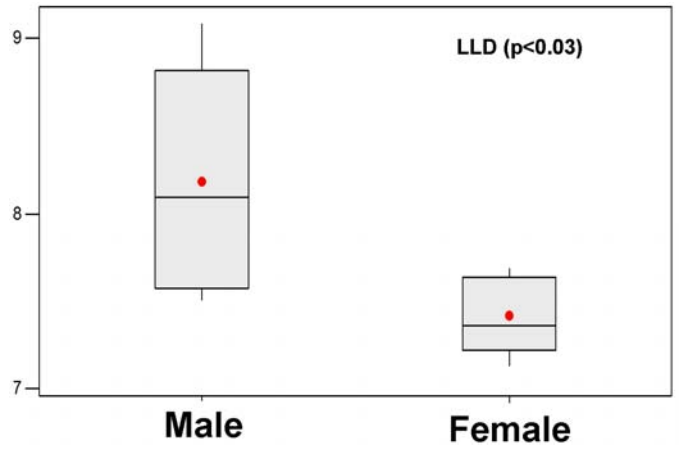
Autopsy has been considered as the gold standart in studies regarding heart hypertrophy and it has been



Graph 1. Cut surface area (CSA) (p<0.008) Paraffin groups.

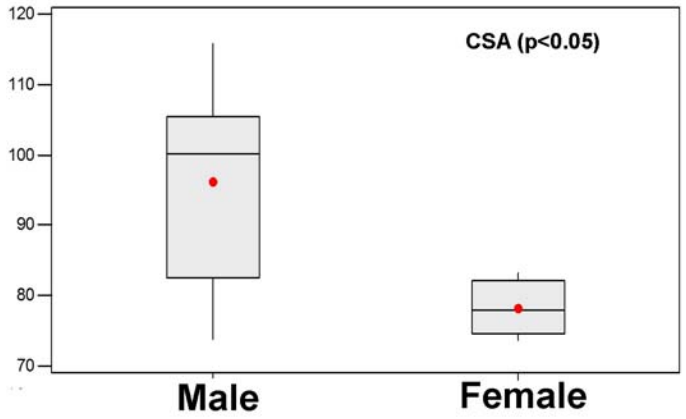


Graph 2. Cranio-caudal diameter (CCD) (p<0.002) Paraffin groups.



Graph 3. Latero-lateral diameter (LLD) (p<0.03) Paraffin groups.

stated that the most objective results of LVM are obtained by this way (Foppa et al., 2005). In studies regarding



Graph 4. Cut surface area (CSA) (p<0.05) Formalin groups.

postmortal heart hypertrophy, the formulation based on Devereux and Reichek (1977) has been commonly used for the morphometrical measurement of the heart. This formulation has been applied on humans and experimental animals and the calculations have been done on LVM and LVMI (Casiglia et al., 2004; Fuster et al., 2005; Grandtner et al., 1974; Kasikcioglu et al., 2007; McAdamms et al., 2010; Noszczyk-Nowak et al., 2009; Ozdemir et al., 2013). In the present study, the evaluations were based on LVM and LVMI calculations too.

In experimental studies concerning the examination of the heart, postmortem measurements (prior to fixation) were not carried out due to certain reasons such as time differences between the time of death and measurements moment and due to the rigor mortis. In the study, the measurement were performed at the histopathological processing steps that consist of fixation, paraffin embedding and sectioning in microtome (Luna, 1968) in order to determine which step was more suitable.

In studies investigated, postmortal ventricular hypertrophy, the echocardiography, ruler and caliper usage and digital image analysis methods were used for heart measurements after formalin fixation (Cavalcanti and Duarte, 2008; Grandtner et al., 1974). In Ozdemir et al. (2013), where the effect of metenolone enanthate on morphometry of the heart were researched, the data were obtained by the method of digital image analysis on photographes taken from paraffin embedded tissues. Heart measurements in two different adult rat strains were done after staining the slides prepared from parafin blocks (McAdamms et al., 2010). In the present study, photos were taken firstly from formalin fixation tissue, later parafin embedded tissues and then from stained slides. LVM and LVMI values were calculated by the measurements obtained from these photos.

Tissues usually somewhat shrink during formalin fixation and paraffin embedding (Luna, 1968). In the present study the lowest values were seen in the Paraffin group and thus it was concluded that the paraffinization

has caused shrinkage more than formalin fixation in heart muscle. In another study conducted on rabbits, the histomorphometrical measurements of heart were lower than supravital measurements done by Electrocardiography (ECG) (Noszczyk-Nowak et al., 2009).

When LVM and LVMI values were considered, there were significant differences between Formalin and Paraffin groups whereas Slide group was similar to them and the values of it were between these two groups. It was commented that, in all groups LVM and LVMI values were higher than those reported by Ozdemir et al. (2013) and the values of LVPT, IVST and RVT were higher than those reported by McAdams et al. (2010).

Conclusion

It can be said that in case report investigation, left ventricular hypertrophy by the help of LVM and LVMI values, formalin fixed tissues and slide measurement findings were more useful, but in experimental study which includes healthy control groups, all three methods might be used. Additionally, it was discussed that the gender has no effect on evaluation among groups in terms of LVM and LVMI. Also the data can possibly contribute to studies that will be conducted on adolescent rats.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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