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Comparison of Different Methods to Measure Experimental Chronic Infarction Size in the Rat Model

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Summary

Objective: To evaluate the differences between three methods for the measurement of experimental infarction in rats in comparison to the traditional method.

Methods: Histological analysis of the infarction area (AREA), histological analysis of the internal cavity perimeter (PER) and echocardiogram analysis of the internal perimeter (ECHO) were compared to the traditional method (histological analysis of the epicardial and endocardial circumferences of the infarction region - CIR). Repeated ANOVA measurements were used in conjunction with the Dunn multiple comparison test, the Bland and Altman concordance method and the Spearman correlation test. Significance was established as $p < 0.05$.

Results: The data of 122 animals were analyzed, 3 to 6 months after the infarction. Infarction size assessments revealed differences between CIR and the other three methods ($p < 0.001$): CIR = 42.4% (35.9-48.8), PER = 50.3% (39.1-57.0), AREA = 27.3% (20.2-34.3), ECHO = 46.1% (39.9-52.6). Therefore, measurement by area underestimated the infarct size by 15%, whereas the echocardiogram and histological internal perimeter measurements overestimated the infarct size by 4% and 5%, respectively. In relation to ECHO and PER, even though the difference between the methods was only 1.27%, the concordance interval ranged from 24.1% to –26.7%, suggesting a low level of concordance between the methods. In relation to associations, statistically significant correlations were found between: CIR and PER ($r = 0.88$ and $p < 0.0001$); CIR and AREA ($r = 0.87$ and $p < 0.0001$) and CIR and ECHO ($r = 0.42$ and $p < 0.0001$).

Conclusion: Despite the high level of correlation, there was a low level of concordance between the methods to define infarct size. (Arq Bras Cardiol 2007;89(2):83-87)

Key words: Myocardial infarction/physiopatology; rats.

Introduction

The experimental acute myocardial infarction model has been widely used for some years now to study physiopathological alterations that occur during and after ischemic attacks.

Among the various models in existence, the experimental infarction in rat models is the most prominent. This is largely due to the fact that it is practical, owing to its relatively low cost in comparison to other animal models, but is mainly due to the reproducibility of results in comparison to subsequent clinical studies[1,2].

One of the most relevant aspects of this model is the infarction size. It is accepted that the acute hemodynamic consequences of a coronary occlusion are closely related to infarction size[3-6]. Chronically, alterations in the composition, mass, volume and cardiac geometry can occur that are collectively called cardiac remodeling[7-9]. The degree of ventricular remodeling is directly

associated with a worse diagnosis due to a greater incidence in the formation of aneurisms, ventricular rupture, arrhythmias and the association with progressive ventricular dysfunction. Similar to the acute alterations, the infarct size is the main determining factor for the presence and intensity of ventricular remodeling after acute myocardial infarction (AMI)[7-9]. Therefore, defining the infarction size is a critical point in the study of morphological and functional repercussions after infarction.

In experimental studies, infarction size has been preferentially evaluated by four different methods: 1) definition of the infarction area in relation to the left ventricle area using histology or planimetry (with planimetry, the infarction area and total myocardial area are evaluated using transillumination); 2) histological analysis to define the internal perimeter of the infarcted region in relation to the total cavity perimeter; 3) histological analysis to define the epicardial and endocardial circumferences of the infarcted and noninfarcted segments and 4) echocardiogram to define the internal perimeter of the infarcted region in relation to the total cavity perimeter. The potential limitation of these four different techniques is that the infarction size can vary depending on the method used. However, the exact degree of variability for each technique has not yet been determined. Therefore, the objective of this

study was to analyze the differences in infarction size estimates using the four different methods.

Methods

The experimental protocol for the present study was approved by our institution's Ethics Commission for Animal Experiments, in accordance with the Ethical Principles for Animal Experiments adopted by the Brazilian College of Animal Experiments.

Experimental infarction - Male Wistar rats weighing between 200 and 250 grams were used for the experiment. The acute infarction was produced in accordance with the previously described method[10,11]. In short, the rats were anesthetized with ether and undewent left lateral thoracotomies. After exteriorization of the heart, the left atrium was separated and the left coronary artery was ligated with #5.00 mononylon thread between the exit point of the pulmonary artery and left atrium. Next, the heart was returned to the chest, the lungs were inflated with positive pressure and the thorax was closed with #10 cotton sutures.

The animals were kept in cages during recovery, received standard commercial feed and were allowed free access to water in a room with controlled humidity and temperature, approximately 25°C, and a 12 hour light - dark cycle.

Echocardiograph Study - After an observation period of 3 to 6 months, the surviving animals (n = 122) were anesthetized with cetamine chloride (50 mg/kg) and xylidine chloride (1 mg/kg) that was injected in the muscle for the echocardiograph study[11]. After trichotomy of the anterior chest region, the animals were placed in the supine position in a specially designed channel that enabled slight lateral left rotation for the test that was conducted using Philips equipment (model TDI 5500) with an electronic transducer with a multi-frequency range of up to 12 MHz. Measurements of the cardiac structures were taken from the M-mode images, using the two dimensional image to direct the ultrasound beam in the parasternal short axis position. All measurements were taken in accordance with the recommendations of the American Society of Echocardiography[12] and previously validated in the infarcted rats model[13]. Infarction size was estimated by defining the internal perimeter of the infarcted region (hypokinetic/akinetic segment) in relation to the total cavity perimeter (ECHO). These values were measured in the two dimensional mode using planimetry in two parasternal views: long and short axis, evaluated in the medial plane.

Morphometric study - After the echocardiography study, the animals were sacrificed, their hearts were removed and dissected, and the right and left ventricles, including the interventricular septum, were separated. Cardiac tissue samples were fixed in a 10% formaldehyde solution for 48 hours using a previously described method[14,15].

The histological sections were stained on a slad with Hematoxylin-Eosin (HE) and Masson agents to assess the infarction tissue, using a LEICA DM LS microscope attached to a video camera that sent digital images to a computer equipped with the image analysis program, Image Pro-plus (Media Cybernetics, Silver Spring, Maryland, USA).

The infarction size was determined in sections 5 to 6 mm from the apex, since the values in this region correspond to the mean of the values obtained from sections of the entire heart[16,17]. Three histological analysis techniques were used to estimate the infarction size: definition of the infarction area in relation to the total area of the left ventricle (AREA), definition of the internal perimeter of the infarction region in relation to the total cavity perimeter (PER) and definition of the epicardial and endocardial circumferences of the infarcted and noninfarcted segments (CIR). The echocardiogram and histological infarction sizes are expressed as a percentile of the ventricular circumference measurements.

Statistical analysis - The CIR method was used as a reference to determine infarction size, since it is regarded as the most reliable method to estimate the infarction region[6]. Therefore, the CIR measurements were used as a reference to compare the following values: CIR *vs* AREA, CIR *vs* PER and CIR *vs* ECHO. The statistical procedures adopted were: variance analysis for repeated measurements, to compare the median values, as well as the Dunn multiple comparison test. Significance was established as $p < 0.05$. The Spearman correlation test and Bland & Altman concordance method were used to establish the concordance intervals between the results for the infarction sizes obtained by the two different techniques.

The Bland & Altman method is a statistical and graphical procedure to compare two methods used to measure the clinical variables. The procedure is as follows: the difference between the measurements obtained from the two methods as well as the mean and standard deviation of these differences are calculated. If the values of the differences have a normal distribution, it is expected that 95% of these values will be between the mean ± 2 SD. This interval is called the "concordance limit". If this interval presents a significant range of variation, it is presumed that there is no concordance between the two methods. Visual examination of the graph reveals significant data dispersion. If there was concordance between the two methods, the difference values would be located close to the zero mark.

Linear regression analysis was used for the correction equations. Values of "0" were given to the infarctions classified as small, when the % value of the infarction was less than 40% for the different methods and values of "1" were given to the infarctions classified as large, when the % value of the infarction was greater than or equal to 40%. Therefore, the sensitivity and specificity values for the methods AREA, PER and ECHO were calculated in relation to the CIR method.

Results

We analyzed the data of 122 animals. The data in regard to the infarction sizes obtained using the different techniques are presented as medians and interquartile intervals. Significant statistical differences were found between CIR and the other three methods (fig.1). As a result, the degree of difference between the area measurement methods, underestimated the infarction size by 15%. In turn, the echocardiogram and histological internal perimeter measurements, overestimated the infarction size by 4% and 5%, respectively.

Comparisons between methods also revealed differences between the AREA methods and the other two techniques ($p < 0.001$). In relation to ECHO and PER no differences
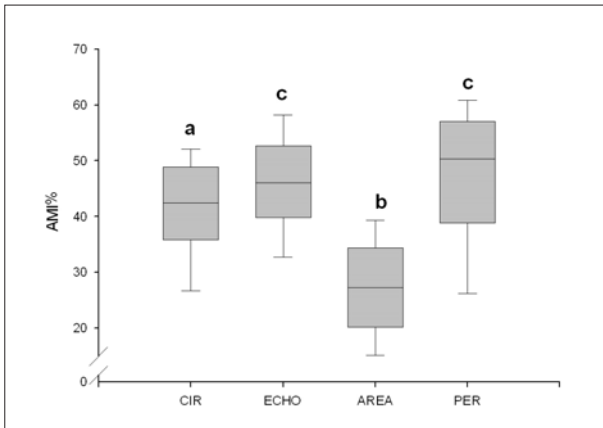
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Fig. 1 - *Differences in defining infarction size using the different methods. CIR - histological assessment of the endocardial and epicardial circumferences of the infarcted segment; PER - histological assessment of the internal perimeter of the infarcted segment; AREA - histological assessment of the area of the infarcted segment; ECHO - echocardiogram assessment of the internal perimeter of the infarcted segment. The different letters represent the statistically significant differences.*
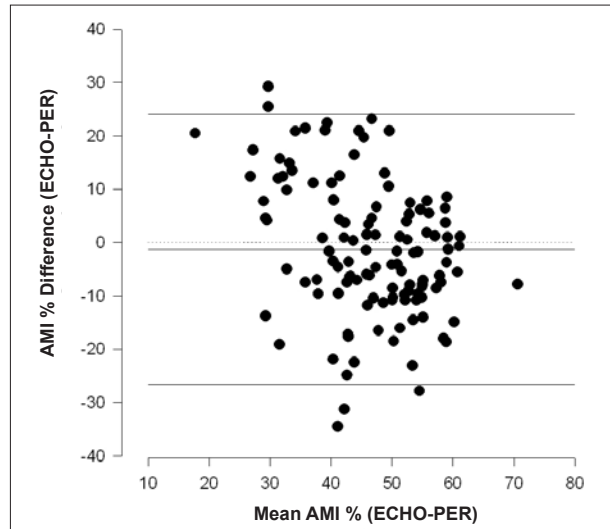

Fig. 2 - *Concordance interval between the methods PER (histological assessment of the internal perimeter of the infarcted segment) and ECHO (echocardiogram assessment of the internal perimeter of the infarcted segment). The differences between the two methods used to obtain the infarct size (PER and ECHO) are plotted on the y axis and the mean result values obtained by the two methods are plotted on the x axis. The concordance limits, or in other words, the mean values of the differences + 2 SD and the mean of the differences - 2SD are shown. In addition, the lines corresponding to zero and the bias (mean of the differences between the methods) is shown.*

were found. Therefore, the next step was to analyze the concordance interval of the two methods. Even though the difference between the methods was only 1.27%, the concordance interval ranged from 24.11% to -26.65%, suggesting a low level of concordance (fig. 2).

In relation to associations between the methods, statistically significant correlations were found between: CIR and PER, with a correlation coefficient of 0.88 and $p < 0.0001$; CIR and AREA with a correlation coefficient of 0.87 and $p < 0.0001$; and CIR and ECHO with a correlation coefficient of 0.42 and $p < 0.0001$ (fig. 3). In addition, we evaluated the influence of the infarction size in the correlations CIR *versus* the ratio of the other methods/CIR. In the comparison between CIR and ECHO/CIR, a strong negative correlation ($r = 0.66$) was found between the methods and infarction size. Therefore, the analysis demonstrated greater variation percentages, or greater

discrepancies between the methods for small infarctions (<30%) suggesting that the infarction size can interfere in the comparison between the different methods (fig. 4). For the other methods, the correlation coefficient values were: PER/CIR ($r = 0.28$) and AREA/CIR ($r = 0.44$).

The linear regression analysis made it possible to prepare equations in order to correct the values obtained in defining the infarction size, using different methods in relation to the CIR method. For the estimated infarction size using the internal perimeter, we obtained the equation: CIR = 10.69 + (0.662 x PER), with a determination coefficient of 82%.
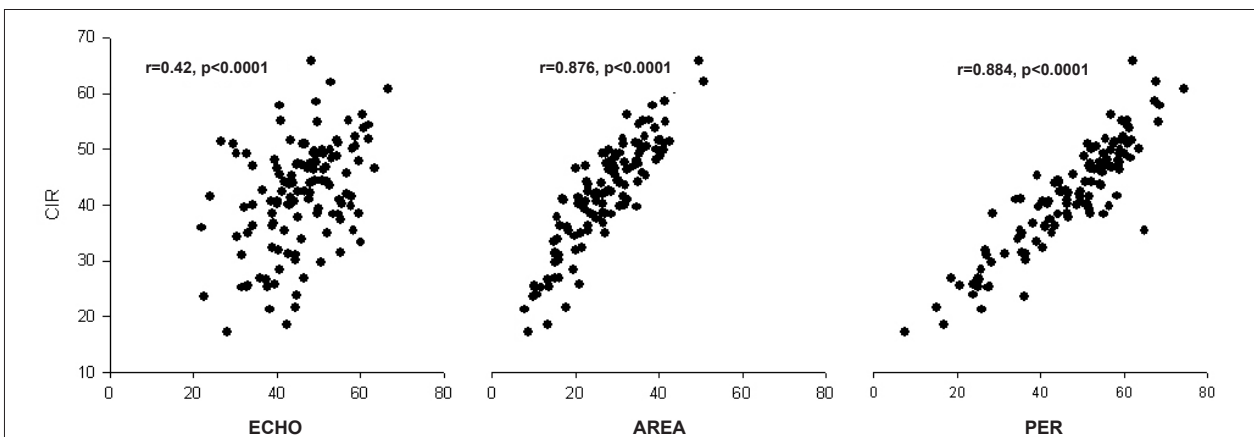

Fig. 3 - *Correlations between the different methods used to determine the infarct size. CIR - histological assessment of the endocardial and epicardial circumferences of the infarcted segment; PER - histological assessment of the internal perimeter of the infarcted segment; AREA - histological assessment of the area of the infarcted segment; ECHO - echocardiogram assessment of the internal perimeter of the infarcted segment.*
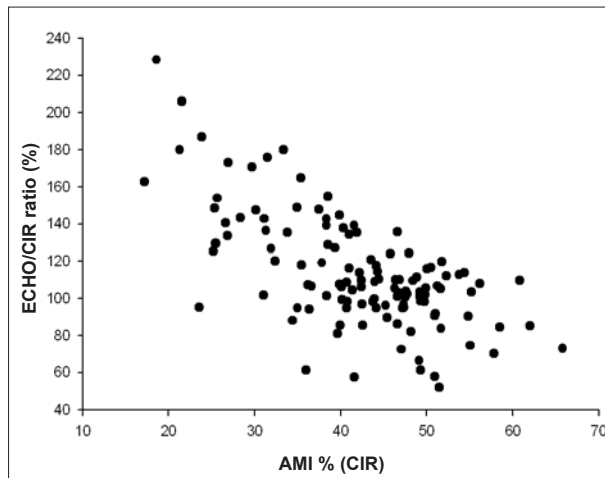
Fig. 4 - Influence of the infarct size on the discrepancy between the methods. Correlation between CIR (histological assessment of the endocardial and epicardial circumferences of the infarcted segment) and the ratio ECHO (echocardiogram assessment of the internal perimeter of the infarcted segment)/CIR.

For the infarction size estimated by the echocardiogram, we obtained the equation: CIR = 21.81 + (0.437 x ECHO), with a determination coefficient of 17%; for the infarction size estimated by area, we obtained the equation: 16.80 + (0.929 x AREA), with a determination coefficient of 78%.

In the sensitivity and specificity analysis in comparison with CIR, the values found were, respectively: ECHO (87%, 46%), PER (96%, 65%) and AREA (14%, 100%).

Discussion

The objective of this study was to compare the different methods to determine infarction size during the chronic phase in the experimental rat model. Our results suggest that, despite the good correlation, there is no concordance between the different methods.

One of the methods used to define the infarction size in the rat model was the analysis of the infarction area in relation to the total area of the left ventricle. Nevertheless, we should consider that after the infarction, dynamic alterations occur in both the infarcted and noninfarcted segments. In the infarcted region, the necrotic tissue is substituted by a fibrous scar tissue. In this process, that lasts 21 days in the rats, the necrotic tissue is reabsorbed and collagen is deposited. In the later stages of healing, the fibrotic area contracts[18]. In the noninfarcted area of the left ventricle, varying degrees of cardiac hypertrophy occur, to compensate for the loss of myocytes. Due to these alterations, the prevailing concept is that definition of infarction size using volume or area can result in errors. In fact the reabsorption and retraction of the infarcted area, added to the hypertrophy in the noninfarcted area, could result in underestimation of the infarction size when compared to the original infarction area[6]. Our data agree with this concept, since, by area, there was a difference of 15% less in relation to the infarction size evaluated using the endocardiac and epicardiac circumferences.

Another method used to determine the infarction size, was the measuring of the internal perimeter of the infarcted segment in relation to the total perimeter of the ventricular cavity. This analysis can be made using two methods: echocardiogram and histology. Nevertheless, in the case of estimations by area, these methods can present significant limitations. In conjunction with the myofibrilla necrosis, the interfibrillar collagen disintegrates. This fact causes loss of connective tissue, making the region more susceptible to distensions and consequent deformations. Therefore, the necrotic muscle areas could shift, realigning the myocytes on the infarcted wall. As a result, the region becomes thinner and the infarcted region dilates. This acute dilatation, marked by the thinning and distension of the infarcted zone, is called infarct expansion[19]. One aspect to be considered is that, secondary to the expansion, the definition of the infarction size using the internal perimeters can overestimate the infarct size[6]. Another interesting finding in our study was that, when compared to the CIR method, the echocardiogram presented greater discrepancies for small infarctions. Additionally, in relation to the echocardiogram, the surgical procedure can cause some artifacts, mainly adherences, that could identify hypokinetic segments that are not related to the infarction.

In agreement with this concept, our results demonstrate that the infarction size was overestimated with both the echocardiogram and histology. An interesting point is that the overestimation of the infarct size using the perimeters (echocardiogram 4% and histology 5%) was less pronounced than the underestimation with the area method (15%).

Another aspect to be considered, is the fact that when comparing the methods, statistically significant correlations with a high degree of correlation coefficients do not ensure concordance between the methods. As an example, we can cite the comparisons between CIR and AREA. Our study found a high degree of correlation coefficients between the methods (r=0.87) and a high significance level (p < 0.0001). We can construe that these techniques presented the same behavior; however we cannot infer that the methods are similar since there was no concordance between the values obtained using the different methods.

Finally, we should consider that in this model, the infarction size can range from 5% to 60%, making it difficult to interpret the repercussions of the infarction. Using the circumference method, Pfeffer and associates[6] related the infarction size to the presence of cardiac remodeling and ventricular dysfunction. For infarctions less than 20%, the animals did not present remodeling or evidence of ventricular dysfunction. The animals with moderate infarctions, between 20% and 40%, presented various degrees of remodeling and ventricular dysfunction, but did not present clinical signs of heart failure. The animals with large infarctions presented significant remodeling and ventricular dysfunction with clinical signs of heart failure. For this reason, when studying this model, the authors usually separate the rats into groups with small (<40%) or large (>40%) infarctions. In our study, the method that presented the best capacity to distinguish between infarctions less than or greater than 40%, in comparison to the traditional method, was PER that presented sensitivity of 96% and specificity of 65%.

Other studies have compared the estimated infarction size using two methods (echocardiogram and histological methods). In a groundbreaking study, Litwin and associates[20]

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did not find any differences in the infarction sizes evaluated by echocardiogram and histology. In the mice model, as in the case of our study, a high correlation was found between the infarction sizes evaluated by histology and echocardiograms[21]. Recently, Nozawa and associates[22] using the rat model, found a high correlation between the echocardiograph and histology methods to estimate infarction size ($r = 0.88$). Nevertheless, as in the case of our study, the Bland-Altman analysis demonstrated low concordance between the two methods, with a concordance interval ranging from -51.4% to $+42.9\%$[22]. Therefore, to date the evidence suggests that despite the "high" correlations, concordance between the two methods has not been identified. Another aspect to be considered is that we did not find any previous studies that compared the four different methods evaluated in the present study.

Our results should be interpreted considering potential limitations. The first distinction is the possible difference between the *post-mortem* and *in vivo* measurements. Measurements of the intact animal are influenced by the pressure of ventricular distension caused by diastolic and coronary perfusion pressures. Another distinction is the potential effect of the fixation in the morphometric methods, since the scar retraction could be different from the normal muscle and distort the area evaluated. These problems could be overcome with some degree of myocardial stretching before fixing the heart, however this was not done in the present study. Additionally, we did not prepare echocardiogram data for intra and interobserver variability.

As shown, the infarction size values present a low level of concordance between the different methods. This fact should be considered when interpreting studies that associate morphological and functional alterations after the ischemic lesion and the infarction size.

In closing, despite the high correlation in the definition of infarction size using the experimental rat model, we found a low level of concordance between the methods.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

There were no external funding sources for this study.

Study Association

This study is not associated with any graduation program.

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