Validation of ultrastructural analysis of mitochondrial deposits in cardiomyocytes as a method of detecting early acute myocardial infarction in humans

Mark P.V. Begieneman¹,²,³, Frank R.W. van de Goot¹,²,³, Jan Fritz¹, Rence Rozendaal¹, Paul A.J. Krijnen¹,³, Hans W.M. Niessen¹,³,⁴

Departments of Pathology¹, Cardiac Surgery⁴, VU Medical Center, Amsterdam, the Netherlands.
Netherlands Forensic Institute², The Hague, the Netherlands.
ICaR-VU³, Amsterdam, the Netherlands.

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Chapter 2

ABSTRACT

Objective: In the present study, ultrastructural analysis of mitochondrial deposits (black dots within mitochondria) as a method for the detection of early acute myocardial infarction (AMI) was evaluated.

Methods: In 24 patients with AMI and six controls, analysis was performed in the heart of infarcted patients and noninfarcted controls.

Results: In the infarction area in lactate dehydrogenase (LDH)-diagnosed AMI, the percentage of positive mitochondria was significantly higher compared to corresponding heart tissue in control patients and compared to noninfarcted areas within these patients. Also in patients with a clinically diagnosed AMI but no LDH decolourisation, a significant higher percentage of positive mitochondria was found in the left ventricle compared to controls and noninfarcted areas.

Conclusions(s): In patients with AMI, an increase in mitochondria with deposits was found in the infarction area compared to controls and noninfarcted tissue within the same patient, suggesting that electron microscopical changes in mitochondria can be used for the diagnosis of AMI less than 3 hours old.
INTRODUCTION

In routine clinical pathological examination during autopsy, but also in forensic pathology, it is important to identify or rule out acute myocardial infarction (AMI) as a cause of death. This is because AMI remains a leading cause of mortality in the Western world. In a macroscopic level, AMI can be identified using a nitro blue tetrazolium staining method, and by doing so, a decreased staining intensity identifies lactate dehydrogenase (LDH) leakage and thus infarction areas. However, LDH decolourisation is only possible from 3 hours after onset of AMI onward. Routine histochemical analysis can only identify infarctions beyond those 3 hours of AMI duration.

Ultrastructural analysis of the heart has been used as a method to identify early infarctions. In the ischemic canine heart ultrastructural changes in mitochondria, including swelling of mitochondria, disorganized cristae, and formation of small osmiophilic amorphous densities, which are composed of lipids and possibly proteins, were detected at 2 hours after onset of AMI. In rats, these ultrastructural changes in the mitochondria were observed as early as 1 hour after onset of AMI. Also in humans, it was shown that AMI induced damage to mitochondria. The ability to use electron microscopic changes for the early detection of AMI in ischemic canine hearts has been questioned, as the same ultrastructural changes have been associated with autolysis. The same was found in rats. These autolytic effects were also shown in human heart tissue, where mitochondrial deposits could be detected as early as 30 minutes postmortem.

In the present study, we analyzed whether quantitative differences exist in the amount of mitochondrial deposits in cardiomyocytes between infarction and noninfarction areas in the heart after AMI and nonAMI hearts in autopsy material and if so whether these can be used to define early infarcts (earlier than 3 hours after onset of AMI) in human autopsy.

MATERIALS AND METHODS

**Human heart tissue**

Human hearts were obtained at autopsy (n = 30) as soon as possible but at least within 48 hours after death. All patients were seen by medical personnel prior to death. In addition, a clinical diagnosis of AMI was made prior to autopsy. Lactate dehydrogenase staining (LDH) (it has to be noticed that tetrazolium used for LDH is harmful) was performed to indicate acute myocardial infarction (AMI), decolourisation indicates affected myocardium. In patients without LDH decolourisation indicating affected myocardium, AMI was clinically defined by ECG. In those patients, left ventricular heart tissue was sampled from suspect areas related to corresponding atherosclerotic changed coronary arteries at risk and/or signs of older infarctions (replacement fibrosis).
In patients with AMI, tissue from the infarction area (left ventricle (LV)) was analyzed via electron microscopy, while in controls corresponding areas of the heart were analyzed. In addition, tissue from the right lateral ventricular wall of the heart from all subjects was analyzed. In patients with AMI, this noninfarcted tissue served as an internal control. This study has been approved by and performed according to the guidelines of the ethics committee of the VU Medical Centre, Amsterdam. Use of leftover material after the pathological examination has been completed, is part of the patient contract in our hospital.

**Electron microscopy**

Heart tissue of all patients was analyzed using electron microscopy. Heart tissue was fixed in 4% formalin and refixed in 2% (v/v) gluteraldehyde for 30 min and 1.5% (w/v) osmium tetroxide for 10 min. The tissue was then dehydrated with acetone and embedded in Epon 812. Ultra thin sections were collected on 300-mesh Formavar-coated Nickel grids. The sections were contracted with uranyl acetate and lead citrate and were examined in a Jeol-1200 EX electron microscope. Ten electron microscopy pictures were analyzed per patient; five of the LV and five of the right ventricle (RV) (magnification 7500x). When comparing the mean amount of mitochondria in the LV and RV of control patients, a significant higher mean amount of mitochondria were found in the RV (LV: 131 ± 13 per 30 EM pictures magnification 7500x vs. RV: 216 ± 25 per 30 EM pictures magnification 7500x, p = 0.020). At an ultrastructural level, mitochondrial deposits appeared as black dots within mitochondria (figure 1). Such mitochondria were defined as positive and indicative for irreversible cell damage5. It has to be noticed that those deposits do not differ ultrastructural from deposits formed by autolysis in control patients (figure 1D). Mitochondria with and without deposits were counted separately in each picture. The percentage of positive mitochondria (mitochondria with deposits) in the LV or RV was then calculated as the total score of each specimen.

**Statistical analysis**

Statistical analysis was performed using the SPSS statistics program (windows version 14.0, SPSS Inc., Chicago, IL). Data was analyzed using a repeated measure ANOVA with post hoc Bonferroni tests and paired T-tests. Levene’s test was used for homogeneity of variances. p values ≤0.05 were considered significant. The factor shows the increase of the percentage of positive mitochondria in the LV in comparison to the RV of the same group or LV of another group. The positive predictive (PPV) value was calculated using the internal control (score of RV). We calculated the PPV by scoring the true positive cases (positive for AMI and 1.28 times more deposits in the LV compared to RV) and true negative cases (negative for AMI and 1.28 times more deposits in the LV compared to RV). After that the total amount of true positive cases was divided by the total amount of true positive and true negative cases, times 100%.
Mitochondrial deposits as a method for detecting AMI

Figure 1

RESULTS

Included were 24 patients who died of AMI and six control patients who died from a cause not related to cardiac disease (table 1). In the AMI group, patients were included when no extravascular neutrophilic granulocytes could be detected in the heart indicative for AMI of less than 12 hours old. In 13 patients with AMI, LDH staining showed LDH decolourisation of the affected myocardium indicative of an infarction of 3 hours old or older. These patients with
AMI had infarction of either the left ventricular anterior wall (n = 7), both lateral and posterior wall (n = 2), posterior wall (n = 1), lateral wall (n = 1) or both lateral and anterior wall (n = 2). In these patients, the infarct age was histological determined to be between 3-6 hours (no neutrophilic granulocytes in blood vessels) (n = 12) and between 6-12 hours old (neutrophilic granulocytes in blood vessels but without extravasation)(n = 1)⁵. In the remaining 11 patients with AMI, AMI was clinically diagnosed; however, no LDH decolourisation was observed. In these patients, the infarct age was therefore determined to be <3 hours.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical data of patients included in the study.</th>
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<tbody>
<tr>
<td></td>
<td>Controls (n = 6)</td>
</tr>
<tr>
<td>Male</td>
<td>5</td>
</tr>
<tr>
<td>Age mean, range (in years)</td>
<td>47</td>
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<tr>
<td>Female</td>
<td>1</td>
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<tr>
<td>Age mean, range (in years)</td>
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<tr>
<td>Cause of death</td>
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<td></td>
<td>Epileptic insult (n = 1)</td>
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<td></td>
<td>APE (n = 2)</td>
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<td>Viral Infection Lungs (n = 1)</td>
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In control patients, the mean percentage of positive mitochondria (figure 1A, D) in the LV of the heart was 48 ± 2%, varying from 26% to 68% underlining that indeed due to post mortal autolysis¹⁵, deposits are formed within the mitochondria (figure 2). In patients with a LDH-diagnosed AMI (varying from 3 hours up to 6-12 hours), the mean percentage of positive mitochondria in the infarction area (LV) (figure 1B, C), however, was 64 ± 2%, varying from 52% to 82% (figure 2). The mean percentage of positive mitochondria in the infarction area of patients with a LDH-diagnosed AMI was significantly higher than the mean percentage of positive mitochondria in the LV of control patients (p<0.001, factor 1.33). In patients with a clinically diagnosed AMI without LDH decolourisation at autopsy (< 3 hours old), the mean percentage of positive mitochondria in the infarcted area of these patients with a clinically diagnosed AMI was significantly higher than the mean percentage of positive mitochondria in the LV of control patients (p < 0.001). To compare the percentage of positive mitochondria in patients with AMI (both LDH and clinically diagnosed only) between infarcted tissue and noninfarcted tissue within the same patient, the
percentage of positive mitochondria was determined in the infarction area from the LV and noninfarced tissue from the RV. In both groups of patients with AMI, the mean percentage of positive mitochondria in the infarction area was significantly higher (respectively, factor 1.28 and 1.47) than the mean percentage of positive mitochondria in the RV (64 ± 2% vs. 50 ± 2% for LDH-diagnosed AMI and 61 ± 2% vs. 41 ± 2% for clinically diagnosed AMI, p < 0.001; figure 2). In control patients, the mean percentage of positive mitochondria was also significantly higher in the LV than RV (48 ± 2% vs. 42 ± 3%, p = 0.008). However, this was only a factor 1.15 higher compared to 1.28 and 1.47 in patients with AMI. Also the mean percentage of positive mitochondria in the RV of patients with a LDH diagnosed AMI was significantly higher compared to the RV of control patients (50 ± 2% vs. 42 ± 3%, p = 0.027). However, no significant difference was found between the percentage of positive mitochondria in the RV of control patients and the RV of clinically diagnosed AMI patients.

![Bar chart showing percentage of positive mitochondria in LV and RV for control and AMI patients](image)

**Figure 2**
Comparison between the mean percentages of mitochondria with deposits in the left and right ventricle of patients with acute myocardial infarction (LDH decolourisation and clinically diagnosed, no LDH decolourisation) and control patients. Error bars represent the standard error of the mean, factor represents % of mitochondria with deposits of the LV divided by % of mitochondria with deposits of the RV.


We also analyzed the amount of patients that had a significant increase in the amount of positive mitochondria in the LV compared to the RV in patients with AMI. In patients with a LDH-diagnosed AMI, 10 out of 13 (85%) patients had a significantly higher mean percentage of positive mitochondria in the LV than in the RV, while in patients with a clinically diagnosed...
AMI, 8 out of 11 (73%) patients had a significantly higher mean percentage of positive mitochondria in the LV than RV. We also calculated a positive predictive value using the internal control (scores of the RV), and this was found to be 89.47%, meaning that in 89.47% of the cases the finding absolutely indicates AMI.

We next analyzed whether increased time between death and autopsy (Δt (hours)) correlated with increased mitochondrial deposits because of autolysis in the LV and RV of the heart. Hence, the patients with AMI (LDH diagnosed) were divided into 2 groups: patients with a Δt of ≤12 hours and 13 up to 48 hours. The mean percentages of positive mitochondria in these 2 groups in the LV were 62 ± 1% (≤12 hours) and 66 ± 2% (13-48 hours) respectively, and were not significantly different. However, in the right ventricle the mean percentages of positive mitochondria in these 2 groups were 46 ± 1% (≤12 hours) and 55 ± 3% (13-48 hours) respectively, and were significantly different (p = 0.013). This shows that AMI patients (LDH diagnosed) with a higher Δt do not have a higher amount of mitochondrial deposits in the LV; however, the RV of these patients with a higher Δt does have a higher amount of mitochondrial deposits. The data of the RV indicates that mitochondrial deposits are formed due to autolysis and do increase significantly in accordance with time elapsed between death and autopsy, at least within the Δt investigated here (up to 48 hours). In the LV no significant increase in mitochondrial deposits was found in accordance with time elapsed between death and autopsy.

**DISCUSSION**

In the present study, quantitative ultrastructural analysis of mitochondrial amorphous densities or mitochondrial deposits (positive mitochondria) indicative for irreversible cell damage as a method for the determination of early AMI was evaluated.

We have found that in the infarction area of patients with a LDH-diagnosed AMI, the percentage of positive mitochondria was significantly higher (factor 1.33) compared to corresponding heart tissue in control patients and compared to noninfarcted areas (RV) within patients with AMI. This was found in 85% of the patients. Also in patients with a clinically diagnosed AMI only (no LDH decolourisation at autopsy) a significantly higher amount of positive mitochondria was found in the LV compared to controls and noninfarcted areas (RV) (factor 1.47). This was found in 73% of the patients.

These results thus indicate that ultrastructural analysis of mitochondrial deposits in the heart can be used to define early AMI of less than 3 hours old.

It is known that postmortem autolysis can also cause formation of deposits in mitochondria in the canine, rat, and human heart[10,14,15]. In the present study, an effect of autolysis was also found in control and AMI patients; in both patient groups we have found formation
of deposits in mitochondria in the LV and RV of the heart. However, in patients with AMI (LDH diagnosed), we found an increase in positive mitochondria in the infarction area (LV) compared to noninfarcted area (RV) within the same patient (factor 1.28) and the LV of the heart in control patients (factor 1.33). This thus suggests that besides autolysis, there is an additional effect of ischemia in patients with AMI (LDH diagnosed). By contrast, Ludatscher et al.\textsuperscript{15} found that after 2-18 hours postmortem deposits are found in all mitochondria due to autolysis; however, they did not quantify the amount of mitochondrial deposits and in their concomitant figure of myocardial autopsy material taken 18 hours after exitus not all mitochondria have deposits. Also they describe that these patients died due to various diseases which was not further specified, and they did not describe any effects of these various diseases on the formation of mitochondria in the heart. Also in our control patients, we did not find deposits in all mitochondria (48 ± 2\%) and compared to patients with AMI there was a significant difference in the amount of deposits found.

Furthermore, we also showed that these same patients with AMI with the longest $\Delta t$ (13 up to 48 hours) between time of death and time of autopsy did not have a significant higher percentage of positive mitochondria in the LV compared to patients with a lower $\Delta t$ ($\leq$12 hours). However, in the RV a significant increase was found, indicative that there is an autolytic effect which does increase in time. As is also shown in the control patients, there is an autolytic effect; however, in the LV of patients with AMI, the percentage of mitochondria with deposits because of the AMI may be so high that an additional autolytic effect is too small in comparison to alter the percentage of positive mitochondria.

In conclusion, our results indicate that ultrastructural analysis of mitochondrial deposits is a valid method to detect early myocardial infarction of less than 3 hours old when the amount of mitochondrial deposits is a factor 1.28 larger in the area of the heart suspected of putative AMI, compared with distant, noninfarcted part of the heart of the RV of the same patient, or a factor 1.33 when compared to the noninfarcted LV of a control patient.

According to literature, the detection of deposits in mitochondria can be seen at earliest at 2 hours post AMI\textsuperscript{10}. This ultrastructural analysis is a particular useful method in cases of sudden or unexplained death in case LDH staining is not conclusive at autopsy. Electron microscopy then can contribute significantly in diagnosing an AMI in between 2 and 3 hours old, thus before LDH decolourisation. A diagram when to use LDH staining and EM analysis for diagnosing AMI is depicted in figure 3.

When using this method, heart samples should be collected during autopsy from suspected areas of the heart related to corresponding atherosclerotic changed coronary arteries at risk and/or signs of older infarctions (replacement fibrosis). It is also recommended to take samples from noninfarcted tissue from the same patient to indicate the “background” signal caused by autolysis. Suspected infarcted and noninfarcted tissue should then be compared to determine a possible AMI. The costs for electron microscopy analysis in the Netherlands is approximately 250 euro (330 US Dollars).
However, our results also show that this method is not absolute and that in our study, in approximately 25% of the patients with an infarction of less than 3 hours old we could not detect evidence for myocardial infarction at an ultrastructural level, although a sampling error never can be excluded in those cases.

**Forensic autopsies**

Is the body in state of decomposition (no green color and/or no body stiffness)?

- **Yes**
  - No LDH staining and EM analysis are performed.

- **No**
  - In all other cases LDH staining is always performed.

  - Clear LDH decolourisation
    - No EM analysis is performed, AMI is diagnosed of at least 3 hours old.
  - No clear LDH decolourisation

  - EM analysis is performed, unless a clear cause of death is found (e.g. shot injury).

  - Number of positive mitochondria: suspected area vs control area (within the same patient): $< \text{factor 1.28}$
    - No AMI is diagnosed.
  - Number of positive mitochondria: suspected area vs control area (within the same patient): $\geq \text{factor 1.28}$
    - AMI is diagnosed of at least 2 hours old.

**Figure 3**

REFERENCES