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Biochemical analysis and immunohistochemical determination of cardiac troponin for the postmortem diagnosis of myocardial damage

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Summary. Cardiac disease is the most common cause of sudden death in Western nations. In forensic practice there is a need for more sensitive diagnostic methods for the postmortem diagnosis of myocardial damage. The aim of this study was to analyse the diagnostic efficacy of biochemical markers in cadaver fluids in conjunction with histological studies and the immunohistochemical determination of cardiac troponin C (cTnC) and cardiac troponin T (cTnT) levels in myocardial tissue fixed in formol and included in paraffin. We studied 50 cadavers (43 males and 7 females) with a mean age of 47.5 years (SD 19.2; range 12 to 87 years). Cases were chosen according to the postmortem interval, cause of death, and circumstances of death. Pericardial fluid and serum were tested in duplicate for cardiac troponin I (cTn I), myoglobin and CKMB by immunoassay system using commercial kits. In myocardial tissue, histological studies were performed with hematoxylin and eosin (H&E), Masson's trichrome staining and immunohistochemical techniques involving streptavidin-biotin-peroxidase were performed. The results pointed to statistically significant differences for all the biochemical markers in pericardial fluid. The highest levels were obtained in the group of cadavers who had died from myocardial infarction. The immunohistochemical expression of cTnC was detected in 86% of cases; it was strongly positive and usually diffuse. The expression of cTnT, was much less frequent (46% of cases) and less intense. It was concluded that the immunohistochemical determination of cTnC and cTnT levels in myocardial tissue may be used as an index of myocardium damage.

Key words: Postmortem, Myocardial, Damage

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Introduction

Acute myocardial infarction is the most common cause of sudden unexpected death. Occasionally, when sudden death occurs in a very early stage of infarction, the lack of outstanding features at autopsy, the presence of unspecific lesions and the difficult detection of myocardial lesions by traditional macroscopic examinations or routine histological stains make it difficult to explain the cause of death. In forensic practice there is a need for more sensitive diagnostic methods for the postmortem diagnosis of myocardial damage. To make things more difficult, the anatomopathological study of the heart of individuals suffering accidental death with cardiac traumatism may reveal similar morphological alterations (edema and necrosis, for example) to those found in myocardial infarction (Tenzer, 1985). This morphological finding, adding more importance to the analysis of biochemical markers (Luna et al., 1982; Janssen, 1984; Stewart et al., 1984; Lachica et al., 1988; Pérez Cárceles et al., 1995, 2004; Osuna et al., 1998a,b). There is a growing trend to study myofibrillar proteins from the cardiac muscle in the search for markers that show a high specificity for cardiac necrosis. In previous studies we pointed to the usefulness of determining cTnI in the pericardial fluid for diagnosing myocardial infarction (Osuna et al., 1998a,b). On the other hand, the structure of cTnC and cTnT in the cardiac muscle has led to the development of an immunohistochemical analysis of both cTnC and cTnT using especially specific monoclonal antibodies (Katus et al., 1992; Larue et al., 1993; Bodor et al., 1995; Antman et al., 1998).

The aim of this study was to analyse the postmortem diagnostic efficacy of biochemical markers in cadaver fluids in conjunction with histological studies and immunohistochemical determination of cardiac troponin C (cTnC) and cardiac troponin T (cTnT) levels in myocardial tissue using material fixed in formol and included in paraffin for determining the cause of death.

Materials and methods

A total of 50 hearts from routinely performed forensic autopsies were studied. The hearts came from 43 males and 7 females, with a mean age of 47.5 years (SD 19.2; range 12 to 87 years). The study was approved by the Ethics Committee of the Institute of Forensic Medicine and the Ethics Committee of the University of Murcia. Cases were chosen according to the postmortem interval, cause and circumstances of death. According to the information obtained from family witnesses, the scene of death and autopsy data we were able to gather information concerning the moment of death in order to calculate the postmortem interval (the time elapsing between death and autopsy). We included only cadavers with a postmortem interval of less than 24 hours. The mean postmortem interval was 6.58±0.49 hours (SD 3.48; range 2 to 16 hours). To minimise postmortem artifacts, the bodies were refrigerated. The average interval between death and refrigeration was 3 hours. Data concerning the initial causes of death and autopsy records were unknown to the persons who performed the biochemical and histological analyses. Cases were assigned to one of four diagnostic groups based on the cause of death according to the patient's medical records, scene of death, autopsy, and toxicological and complementary histological findings. The groups were as follows: (1) myocardial infarction (n=14) (9 deaths witnessed); (2) asphyxia (n=8) (5 cases of hanging and 3 of drowning); (3) multiple trauma (n=12), (all motor vehicle collisions witnessed and with chest trauma); (4) natural deaths except myocardial infarction (n=16, all deaths witnessed) (9 cases of cerebrovascular disease, 2 of pneumonia and pulmonary embolism, 3 of acute renal failure, and 2 of gastric acute haemorrhage). Cardiopulmonary resuscitation had been applied in 18 subjects.

Pericardial fluid and serum from femoral vein were tested in duplicate for Access® cardiac troponin I (cTn I), Access® myoglobin and Access® CKMB (mass) using commercial kits (Beckman Coulter, Inc.) by a quantitative chemiluminescent immunoassay based on a quantitative determination, from paramagnetic particles in solid phase, of the levels of the different biochemical markers. Samples were centrifuged and stored at -80 °C. The myoglobin, cTnI and CKMB (mass) concentrations in serum samples from healthy subjects are below 70 ng/mL, 0.1 ng/mL and 4.0 ng/mL respectively. When initial determinations showed concentrations of markers above the measurement range, the samples were diluted with Access® CKMB Diluent and Access® Sample Diluent A, for CKMB and myoglobin, and saline stabilised with 3% albumin to adjust the concentrations to within the clinical ranges in the case of cTnI.

For the histological and immunohistochemical study, we obtained samples from zones of the heart showing signs of necrosis or hemorrhage (when present), or from the upper third of the intraventricular wall and upper third of the free wall of the left ventricle (those areas where myocardial infarction would most likely be situated). Histological studies with hematoxylin-eosin (H&E) staining and Masson's trichrome staining in formalin-fixed paraffin sections were performed. Immunohistochemical techniques using streptavidin-biotin-peroxidase according to the modificated Hsu' methods (Hsu et al., 1981) were performed. As primary antibodies we used monoclonal antibodies against subunits C and T of troponin (NCL-TROPC and NCL-TROPT, Novocastra Lab Ltd, UK), which react with human cardiac troponin C and human fast muscle troponin T. The monoclonal antibody for subunit I is only suitable for fresh specimens, and did not serve for the controls included in paraffin (as controls we used tissue of normal heart). Because the immunohistochemical study was performed in material fixed in formol and included in paraffin, it was necessary to increase the reactivity of the tissue by means of a pretreatment involving tripsin digestion at 37 °C for five minutes. The working dilution of monoclonal antibodies were 1:40 and 1:20, respectively. For the rest of the immunohistochemical study, we used the LASAB kit (DAKO diag. Barcelona, Spain). The tests were performed by two different observers and were arranged according to the intensity of expression and number of cells stained, using a range of positivity from 0-3 crosses(+): 0, negative; 1+, weak positivity in less than 50% of cells; 2+, weak positivity in 50-100% of cells; 3+, strong positivity in 100% of cells. We were unable to detect troponin I since the material used was fixed in formol and included in paraffin.

Statistically significant correlations were determined between different variables and discriminant analysis was applied. A non-parametric test (Kruskal-Wallis Test) was used to compare groups. Also specific contrasts for each variable grouped according to the diagnostic categories were carried out using the Mann-Withney Test.

Results

The histological findings are summarized in Table 1. Note the signs of necrosis (cytoplasmic eosinophilia and

Table 1. Histological finding in the interventricular wall and in the left ventricle.

LESION	LOCATION	
	Interventricular wall	Left ventricle
Edema	36	39
Congestion	19	10
Haemorrhage	3	6
Inflammation	5	6
Cytoplasmatic vacuoles	15	12
Contraction bands	13	16
Necrosis	38	37
Diffuse fibrosis	24	24

Table 2. Mean, standard deviation (SD) median and range values for the biochemical parameters in the diagnostic groups of myocardial infarction and asphyxia.

	MYOCARDIAL INFARCTION (n=14)				ASPHYIA (n=8)			
	mean	SD	median	range	mean	SD	median	range
cTn I (ng/mL)								
Pericardial fluid	172	267	51.3	2.1-852.8	3.8	8.7	0.39	0.1-25.3
Serum	11.8	23.6	1.02	0.007-86	2.7	5.0	0.18	0.01-12.9
Myoglobin (ng/mL)								
Pericardial fluid	43512	74038	18333	1798-264504	6820	7792	3960	272-214112
Serum	40024	63863	16932	1058-210240	19501	28684	9402	1914-88800
CKMB (ng/mL)								
Pericardial fluid	3011	5161	450.8	15.9-13456	31.2	45.7	12.5	0.3-128.7
Serum	235.6	486.2	81.8	12-1892	62.6	74.1	29.2	11.3-189.4

Table 3. Mean, standard deviation (SD) median and range values for the biochemical parameters in the diagnostic groups of multiple trauma and other natural deaths.

	MULTIPLE TRAUMA (n=12)				OTHER NATURAL DEATHS (n=16)			
	mean	SD	median	range	mean	SD	median	range
cTn I (ng/mL)								
Pericardial fluid	104	236	11.1	0.2-825	6.4	9.8	2.4	0.02-38.8
Serum	5.9	10.5	0.1	0.04-30.9	3.2	8.2	0.45	0.003-32.4
Myoglobin (ng/mL)								
Pericardial fluid	24914	29183	11053	1220-94768	11520	11763	7619	245-46420
Serum	48745	89942	11280	1000-298336	53450	115735	17408	580-457320
CKMB (ng/mL)								
Pericardial fluid	501	1209	154.6	5.7-4312	87.6	105	50.7	4-378
Serum	515	1301	130.4	3.5-4632	346	881	55.9	2.3-3574

Table 4. Kruskal-Wallis Test used for the biochemical values in the diagnostic groups (myocardial infarction, asphyxia, multiple trauma and other natural deaths).

VARIABLE	df	STATISTIC	PROBABILITY
cTn I. Pericardial fluid (ng/mL)	3	18.18	P<0.001
Myoglobin. Pericardial fluid (ng/mL)	3	7.45	P<0.05
CKMB. Pericardial fluid (ng/mL)	3	19.27	P<0.001
cTn I. Serum (ng/mL)	3	4.60	NS
Myoglobin. Serum (ng/mL)	3	0.90	NS
CKMB. Serum (ng/mL)	3	4.11	NS

Groups: 1, Myocardial infarction (n=14); 2, Asphyxia (n=8); 3, Multiple trauma (n=12); 4, Other natural deaths (n=16); df, degrees of freedom; NS, not statistically significant

nuclear retractions) in the left ventricular (37 cases) and in the interventricular wall (38 cases). Contraction band necrosis was found in 16 cases in the left ventricle and in 13 cases in the interventricular wall. Signs of diffuse fibrosis in the interventricular wall were found in 24 cases.

Tables 2 and 3 show the values (mean ± standard deviation, median and range) obtained for cTnI, myoglobin and CKMB in serum and pericardial fluid for the diagnostic groups. A non-parametric test (Kruskal-Wallis Test) was used to compare the values of the different biochemical markers in the diagnostic groups (Table 4). In pericardial fluid, statistically significant differences were observed for all the biochemical markers. The highest levels were obtained in the group of cadavers who had died from myocardial infarction. The next highest levels were found in the group of cadavers from subjects who had died from trauma with chest injuries. No statistically significant differences were obtained in serum. Table 5 shows the concentrations (mean ± standard deviation, median and range) obtained when the sample was distributed into two groups, one of subjects dying from myocardial infarction or multiple trauma (N=26) and the other comprising the remaining cases (N=24). We used this grouping because these two groups comprise those cases who died from myocardial damage, ischemic (myocardial infarction) or traumatic (multiple trauma with chest trauma). Statistically significant differences were observed between the groups (Table 6). The highest

values were found in the group of subjects who had died of myocardial infarction or chest trauma. No statistically significant differences were obtained in serum.

We observed no statistically significant correlation between the levels of biochemical markers in pericardial fluid and the postmortem interval. As regards serum, the

Table 5. Mean, standard deviation (SD) median and range values for the biochemical parameters when the sample was distributed into two groups, one of subjects dying from myocardial infarction or multiple trauma (N= 26) and the remaining cases (N=24).

	MYOCARDIAL INFARCTION OR MULTIPLE TRAUMA (n=12)				OTHER CAUSES OF DEATHS (n=16)			
	mean	SD	median	range	mean	SD	median	range
cTn I (ng/mL)								
Pericardial fluid	140	250	16.8	0.2-852	5.5	9.4	1.5	0.02-38.8
Serum	9.1	18.6	0.58	0.007-86	3.0	7.2	0.3	0.003-32.4
Myoglobin (ng/mL)								
Pericardial fluid	34928	57572	14153	245-46420	9953	10669	5853	245-46420
Serum	44049	75497	13704	580-457320	42133	96225	13289	580-457320
CKMB (ng/mL)								
Pericardial fluid	1853	4015	203.2	0.3-378	68	92	38.7	0.3-378
Serum	364	942	13704	2.3-3574	251	725	42.3	2.3-3574

Table 6. Mann-Withney test used to compare mean values of the biochemical markers in relation to the cause of death (myocardial infarction or multiple trauma and the remaining cases).

VARIABLE	MANN-WHITNEY U	WILCOXON W	Z	PROBABILITY
cTn I. Pericardial fluid (ng/mL)	108.0	408.0	-3.961	P<0.001
Myoglobin. Pericardial fluid (ng/mL)	183.5	483.5	-2.495	P<0.05
CKMB. Pericardial fluid (ng/mL)	122.0	422.0	-3.689	P<0.001
cTn I. Serum (ng/mL)	241.0	541.0	-1.379	NS
Myoglobin. Serum (ng/mL)	296.0	596.0	-0.311	NS
CKMB. Serum (ng/mL)	228.0	528.0	-1.631	NS

Groups: 1, Myocardial infarction or multiple trauma (n=26); 2, The remaining cases (n=24). NS: not statistically significant.

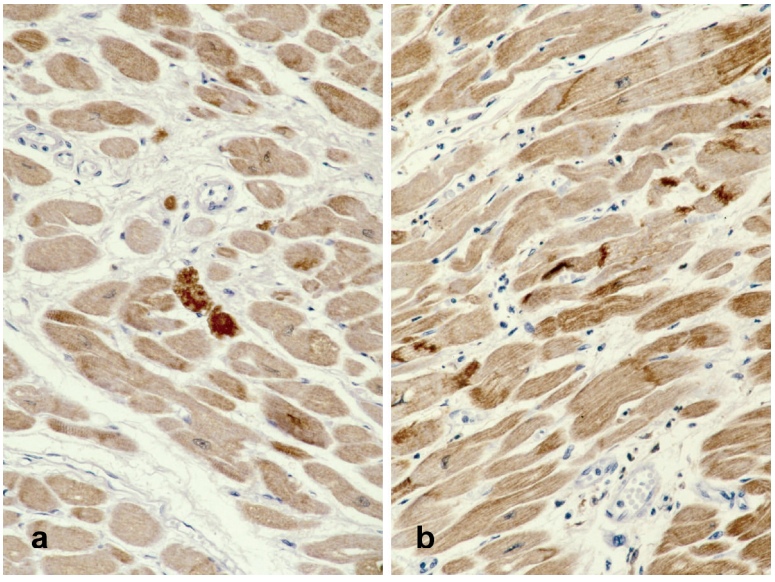

Fig. 1. Immunohistochemical expression of cTnC in isolated cells showing necrosis **(a)** and in the contraction bands **(b)**. a, x 325; b x 300

only statistically significant and direct correlation was between the levels of cTnI and the postmortem interval (P=0.014). We found statistically significant correlations between the diagnostic group and the levels of myoglobin (P=0.002), CKMB (P=0.015) and cTnI (P=0.014) in pericardial fluid. No statistically significant correlations were found between the diagnostic category and the biochemical markers in serum.

The immunohistochemical expression of cTnC was detected in 86% of cases, where it was strongly positive and usually diffuse. Note that it was expressed with special intensity in isolated cells showing necrosis and in the contraction bands (Fig. 1a,b). However in the extensive area of infarction, it was so slightly expressed that it disappeared at times (Fig. 2). cTnT, on the other hand, was much less frequent and was detected in only 46% of cases, and then only in given foci and with a slight intensity.

For discriminant analysis, we chose the diagnostic category as the grouping variable, establishing two groups: cases of death from myocardial infarction or trauma (N=26) and the rest of the cases (N=24). When we included the concentrations of the different biochemical markers analysed in serum and pericardial fluid, correct classification was found in 74% of cases, rising to 100% in the group of cases with no myocardial infarction. If we included in the discriminant analysis the concentration of the different biochemical markers and the results obtained by immunohistochemical analysis, correct classification was achieved in 80% of cases and 91.7% in the group of cases with no myocardial infarction. Only two cases were wrongly classified (a case of pulmonary embolism and a case of drowning), with high concentrations of myoglobin in both fluids and an intense expression of cTnC seen in the immunohistochemical analysis. In the case of drowning, even though the cause of death is asphyxia, hematoxilyn-eosin staining still reveals histopathological findings that are compatible with acute myocardial infarction.

Discussion

The heart-specific troponins are like myoglobin and other muscle protein components of the normal myocardial cells and appear in high concentrations in serum and pericardial fluid after acute myocardial infarction (Mair et al., 1995; Adams et al., 1996; Bertinchant et al., 1996; Cina et al., 1998; Coudrey, 1998; Ognibene et al., 1998; Hansen and Rossen, 1999; Ortmann et al., 2001). In postmortem diagnosis the measurement of these markers is recognized as important for diagnosing myocardial necrosis when such a lesion is suspected but cannot be established by routine histological methods (Stewart et al., 1984; Lachica et al., 1988; Pérez Cárceles et al., 1995). The proper use of a wide variety of biochemical determinations in blood, cerebrospinal fluid, vitreous humor, pericardial fluid, and other fluids can help in solving forensic problems in approximately 10% of the routine natural deaths that comprise the majority of cases seen in any medical examiner's office (Coe, 1993). The interpretation of the results obtained in a biochemical analysis is complex, given the circumstances of the deaths and the autolytic processes that may occur. In our study we used a short postmortem interval (mean 6.58±0.49) and range 2 to 16

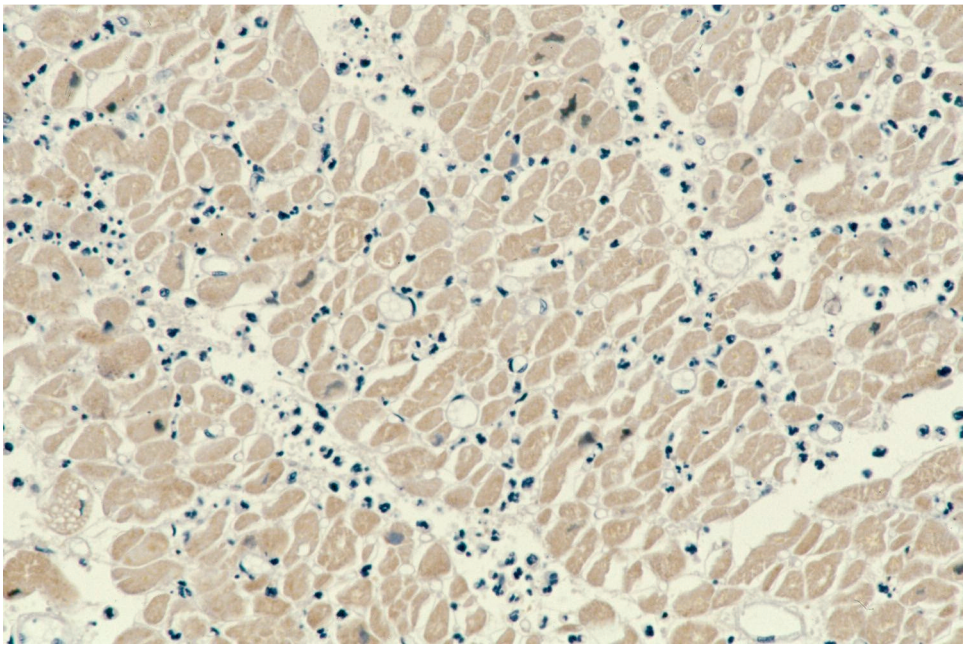

Fig. 2. Immunohistochemical expression of cTnC in the infarcted area. x 200

h. Furthermore, emphasis should also be placed on the short period of time elapsing between death, refrigeration of the body and sample preservation at -80 °C and the time at which the samples were thawed in the laboratory for analysis, thus ensuring their correct degree of preservation and minimising autolytic effects. In our study, only a very weak degree of statistical significance was obtained between postmortem interval and the levels of cTnI in serum. One possible reason for this may be the greater vulnerability of serum to the effects of autolysis, whereas pericardial fluid, for its very composition and situation, constitutes an ideal medium for measuring these concentrations since it is an ultrafiltrate and therefore less subject to contamination (Butany and Woo, 2001). Among its characteristics is its closeness to the cardiac tissue, which gives it special importance in the study of lesions to the cardiac muscle. In our study, we found statistically significant differences between the concentrations of the three markers studied in pericardial fluid, the highest levels corresponding to the group of subjects who had died from myocardial infarction. These results bear out previous findings of ours, confirming the usefulness of these markers in pericardial fluid for postmortem diagnosis (Osuna et al., 1998b; Pérez Cárceles et al., 2004). In addition, pericardial fluid because of its proximity to the myocardium, any alteration in cardiac tissue will be reflected at an earlier stage in pericardial fluid than in serum (Osuna et al., 1998b). The lack of significance in the serum levels of myoglobin and CKMB between the different diagnostic groups may be due to false positive increases attributable to skeletal muscle injury or intense agonic processes. As regards the determination of cTnI in serum, we emphasise once again the possible interference of the postmortem interval. In addition, if the survival time is relatively short, the release of markers into serum will only have been through passive transport processes, while if the heart continues to beat after a cardiovascular or traumatic accident, much higher concentrations of markers will be released into serum, an event that would not occur in pericardial fluid where, diffusion would be a passive process.

In clinical practice, it has been seen that measurement of cTnT and cTnI is more accurate than the conventional measurement of CK-MB (Heeschen et al., 2000; Pagani et al., 2001), and although it has been suggested that cTnT may be of use at autopsy as a qualitative diagnostic test (Cina et al., 2001), great care must be taken in the cases of patients suffering renal failure, where high levels of cTnT may be found (Fredericks et al., 2001). There is general agreement that in serum cTnI is a highly specific marker for myocardial injury. Also, it has also been suggested that cTnI immunoreaction in autopsied hearts is a sensitive test in the diagnosis of early myocardial infarction (Hansen and Rossen, 1999). In a review of data on the accuracy of cTnT and cTnI for the diagnosis of acute myocardial infarction in the emergency department (Ebell et al., 2000) concluded that sensitivity increases for both cTnT and cTnI from 10% to 45% within 1 hour of the onset of pain (depending on the cutoff) to more than 90% at 8 or more hours. Specificity declines gradually from 87% to 80% from 1 to 12 hours after the onset of chest pain for cTnT and is approximately 95% for cTnI. The peak abnormal value in the first 24 hours after admission to the emergency department has an area under the ROC of 0.99 and is very useful at ruling out acute myocardial infarction.

On the other hand Hein et al. (1995) investigated by immunohistochemistry the effects of total myocardial ischaemia in tissue samples from human left ventricles obtained from heart transplant and found that ischaemia causes damage to the contractile proteins sooner than to the cytoskeleton and subcellular organelles.

As we mentioned in Material and methods, the immunohistochemical study was made in material fixed in formol and included in paraffin, so that only monoclonal antibodies against C and T fractions of troponin could be used, while the I fraction of troponin can only be expressed when fresh. The study showed that cTnC expression was almost constant, being especially intense in the contraction bands and in some isolated cells showing necrosis phenomena, while cTnT, which is less associated with the cardiac muscle, was expressed less frequently and less strongly, although in the same zones. This suggests that in cells undergoing apoptosis there is a greater concentration of troponin due to condensation, while in zones of obvious ischemic necrosis (infarction zones) tissue antigens are severely depleted (Ortmann et al., 2000). Earlier studies of acute myocardial infarction found that the immunohistochemical staining of the proteins may be useful as 'negative markers' due to the loss of staining in the infarcted areas (Leadbetter et al., 1990; Martínez-Díaz et al., 2004). Hansen and Rossen (1999) found the same pattern of the cardiac troponin expression.

We are therefore of the opinion that to properly evaluate the cardiac lesions of patients who die suddenly, including cases where death involves thoracic trauma, the procedure to follow is that outlined in this contribution. First the extraction of blood from the vein and pericardial fluid in which we biochemically determine the values of cTnI and CK-MB; then a macroscopic study of the heart, choosing suitable samples for microscopic examination and immunohistochemical staining to detect the C fraction of troponin. Using this procedure, it should be possible to rule out a cardiac cause of death with 100% accuracy. In conclusion, evaluation of the immunohistochemical expression of cTnT and cTnC represents a highly sensitive marker of myocardial lesion.

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