(4) POST MORTEM STUDY OF HEART IN CASES OF SUDDEN CARDIAC DEATH USING TRIPHENYL TETRAZOLIUM CHLORIDE AND HAEMATOXYLIN & EOSIN STAIN

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ABSTRACT:-
Background:
Myocardial infarction (MI) is the most common cause of death for which the largest number of autopsies are done.

Aims:
A clinical study was done to determine those cases numerically smaller but medico-legally more important in which some unnatural event like a road accident, is suspected to die due to acute myocardial infarction. The likelihood of identifying an early infarct minimizes false positivity in cases of sudden death.

Materials and Methods:
A histochemical study on 100 hearts was carried out with using haematoxylin & eosin as a routine stain and Triphenyl tetrazolium chloride as gross marker, the latter can detect infarcts of less than 6 hours of age which help to assess the actual age of the infarct and confirmation of myocardial infarction as the cause of demise.

Results:
68% of cases showed positive result with TTC staining while only 25 % of cases showed positive result with H & E stain. 75% of cases showed the age of infarction within 5 to 8 hours which made possible to detect early infarction by TTC staining. In 82.3% of cases gross examination did not reveal any evidence of infarction, but were revealed by TTC staining.

Conclusion:
Triphenyl tetrazolium chloride is the detector of earliest changes of infarction in comparison to gross and microscopic changes in the infracted heart.

Keywords:
Triphenyl tetrazolium chloride, early infarct.

Introduction:-

Ischemic heart disease (IHD) is the leading cause of death worldwide for both men and women. In more than 90% the cause of myocardial infarction is reduced blood flow due to obstructive atherosclerotic lesion in the coronaries, thus IHD is also termed as coronary artery disease (CAD) or coronary heart disease (CHD).[1] CAD is by far the most frequent cause of sudden and unexpected deaths which constitute a significant portion of autopsies conducted by forensic pathologists in our country, because these deaths appear in apparently healthy without any diagnosed disease that can be attributed to the cause of death, or the period of illness before the supervening of death is so short that the disease cannot be diagnosed early.

The realization of the need for establishing the diagnosis of myocardial infarction in the initial 8 hours, where definite evidence of infarction is lacking has propelled a number of studies on the histochemical, electron microscopic and fluorescent microscopic changes. Also many times significant stenosis of coronary arteries is encountered in deaths due to some other natural disease, in these cases the query always arises whether myocardial ischemia might have contributed to death.[2] With these considerations it was thought worthwhile to study myocardial ischemic changes, with gross histochemical staining and routine staining methods. The histochemical method for studying the enzyme reaction in the myocardium, to differentiate between the healthy myocardium and the infracted myocardium is by far the most sensitive method for diagnosing early myocardial ischemia and is comparatively simple as compared to other methods. The present study has been carried out with utilization of Triphenyl tetrazolium chloride as gross marker and haemotoxylin and eosin as a routine staining method on dead myocardium to separate it from viable myocardium to assess the actual age of myocardial infarction and to confirm myocardial infarction as the only cause of death.

Materials and Methods:-

The present study was carried at the Department of pathology and department forensic medicine and toxicology during the period of August 2009 to August 2011in M. P. Shah medical college, G. G. hospital, Jamnagar. Total 100 cases were studied. The study was approved by institutional ethical committee, prior to inclusion of cases in the study. Detailed history and post-mortem (PM) findings of heart of all cases were collected. PM study was carried out in clinically suspected/ confirmed cases of MI. In addition 3 cases of sudden death due to other causes were also included in the study as negative control. Cases showing signs of decomposition were not included.

Hearts obtained from the test cases were subjected to meticulous gross examination of all three coronary vessels and their major branches by serial transverse sectioning of vessel to note the degree of calcification, stenosis, and presence of thrombus or hemorrhage into an athermatous plaque. This was done by cutting transverse sections of the coronaries and their main branches at 3 mm intervals. Complete transverse slice of ventricular myocardium from sites containing evident or suspected fresh infarct by gross appearance; significant narrowing of vessel supplying the area etc were subjected to TTC macro test. In absence of any of these pointers, multiple slices were studied. Routine microscopic examination by H & E stain of suspected infarct or peripheral area of visible fresh infarct or fresh and old infarction delineated after TTC test was also undertaken.

TTC Macro Test:

Transverse Slicing of Myocardium[3, 4, 5, 6, 7]: 1.5 cm thick transverse slices were cut from ventricular part of fresh heart (without formalin fixation) from apex of heart (Figure 1). This method permits best identification of infarction as well as easy location and documentation of grossly discernible myocardial infarcts. It is indicated whenever ischemic heart disease is in question and quantification of infarct size is desirable. Select the slices which is likely to be a contain infracted area as per guidelines mentioned above were dipped in running water before incubating to remove excess of blood from the surface of the slices but were not allowed to soak or macerate in water as the enzyme reaction is purely a surface phenomenon and prolonged washing elutes the contents of the superficial cell thus giving a false reading. The slices were incubated in 1% solution of 2, 3, 5 Triphenyl Tetrazolium chloride (TTC) for 20-30 minutes, at room temperature or some authors advocate incubation at $37\text{-}40^0$ C. The incubating fluid was prepared by dissolving 1 gm of TTC powder in 100 ml of phosphate buffer at pH 8.5. The staining was carried out in appropriate sized (preferably glass cylindrical of 7.5 diameter and 9.5 cm height) wide mouthed container with screw cap, which can hold a complete transverse slice. During the process of incubation the container is to be closed by its screw cap and kept in a dark place. As exposure to air and light will make the solution lose its potency. TTC solution was freshly prepared every time and was used within half an hour. The slice was to be immersed completely in the solution during the process. The upper surface of it being at least 2 cm below the fluid level. We found that 100ml of 1% solution poured into the above sized container fulfils this criterion, when one slice of heart was immersed into it. The most important precaution of all was to regulate the pH of incubating fluid at 8.5 otherwise the formation of formazan pigments will be unsatisfactory. For better results the slice should be turned over once or twice during the process to discourage stagnation of the reagent and causing excessive deposition of the dye on the surface of the heart slices thereby producing artifacts. The slices were turned over after 15 minutes to prevent prolonged contact of the tissue with the bottom of jar. At the end of the staining procedure the slice was transferred to a jar containing 10% formal saline. This will halt the reaction immediately in addition to fixing the tissue.

Interpretation:

On completion of the staining the infracted myocardium will show up as pale pink colored area (Figure-2, 3) as against the bright red colored normal myocardium (Figure-4). Even old infarcts and scars get delineated (Figure-5). In absence of infarction, both the surface of the slice will show uniform bright red coloration.

Fig 1 Method of dissection of TTC staining transverse slicing of Heart 00

Fig 2 TTC stained heart slice - Fresh infarct seen as pale stained area

Fig 3 TTC stained heart slice - Old infarct in left ventricle & Fresh infarct in right ventricle

Fig 4 TTC stain - Normal myocardium uniformly stained

Fig 5 TTC stained slice - Old infarct seen as unstained area

Results:
Table No. 1   Result of TTC stain and H&E stain of myocardium slice

Age Range	Total	Positive TTC stain	Positive H&E stain	Negative TTC stain	Negative H&E Stain	Doubtful TTC stain	Doubtful H&E Stain
0-10	0	0 (0%)	0 (0%)	0	0	0	0
11-20	3	1 (33.3%)	0 (0%)	1	2	1	1
21-30	3	2 (66.7%)	1 (33.33%)	1	2	0	0
31-40	21	13 (61.9%)	0 (0%)	6	19	2	2
41-50	21	15 (71.4%)	3 (14.28%)	5	17	1	1
51-60	37	25 (67.6%)	15 (40.54%)	11	21	1	1
61-70	10	8 (80%)	4 (40%)	2	6	0	0
71-80	5	4 (80%)	2 (40%)	1	3	0	0
81-90	0	0 (0%)	0 (0%)	0	0	0	0
91-100	0	0 (0%)	0 (0%)	0	0	0	0
Total	100	68 (68%)	25 (25%)	27	70	5	5

Table No. 1 shows early ischemic changes in myocardium were detected by positive TTC staining in 68% of cases. Amongst these 80% results were observed in 7th and 8th decade. Only a case (33.3%) of young adult was shown positive result with TTC staining. In present study only 25 cases out of 100 were detected by H & E stain. 40% of result was obtained in age group 51-80 years indicates low sensitivity of H & E stain in selected sample type and post myocardial infarction survival duration is longer in middle to older age group.

Table No. 2 Correlation of survival time with TTC staining

Period of survival (Age of infarction)	Cases
0-4 hours	04 (5.9%)
5-8 hours	51 (75%)
8-12 hours	06(8.8%)
Not known	07(10.3%)
Total	68

Table No. 2 shows maximum (75%) number of cases showed the age of infarction within 5 to 8 hours which made possible to detect early infarction by TTC staining in comparison to gross and microscopic changes in the heart following myocardial infarction. The period of survival was not known in 7 cases because reliable history was not available.

Table No. 3 Signs of fresh infarct on gross examination

Gross finding	No. of case
Grossly infarct present	12 (17.6%)
Grossly infarct absent	56 (82.3%)
Total	68

Table No. 3 shows maximum (82.3%) cases of which gross examination did not reveal any evidence of infarction, which were revealed by TTC staining. So, TTC is found to be useful test to detect the early infarct as well as to delineate the existed infarct. The periods of survival assessed from the onset of the episode of chest pain to the death, ranged from 1 hour to 12 hours. The attempt was made to correlate the result of TTC with supposed period of survival.

Discussion:-

The absence of gross as well as microscopic changes before appearance of neutrophil at the scene of infarct, which is estimated to take minimum of 6-8 hours in term of post-infarction survival time, has been a major hurdle in establishing the cause of death in cases of early death due to myocardial infarction. As Nitro-Blue and TTC have almost same interference and present study was experimental study, studies regarding Nitro-Blue have also been included because less work has been done on TTC application.

TTC Results:

Present study reveals fresh infarct in 82.37% autopsy cases using TTC stain, which is closely correlates with Agdal[8] study where he found fresh infarct in 75% of cases, indicate more number of fresh infarct can be detected by using TTC.

Grossly Visible Infarct:

In present study there were 68 TTC positive test, amongst them 56 specimen of heart did not show infarction on macroscopic examination. After application of TTC, infracted areas were well visualized. In 12 hearts in which macroscopically infracted area were seen, after application of TTC these areas were better delineated. Finding of the present study is in accordance with Neural et al[9,10], Nachals and Shnikta[5], Ramkissoon[6], Brody et al[4] and Mc Vie[11]

Period of Survival:

Another issue to be considered was the detection of myocardial ischemia in relation to the period of survival and also whether these techniques which outline experimental myocardial infarcts, before the gross or microscopic changes manifest, can be used to assist pathologists in the recognition of early or small infarcts in the human subject.

The earliest infarcts that was detected by TTC methods adopted in present study is of 1 hour duration (old), These observations were closest to those made by Fine[12], where he found loss of enzyme activity from 1½ hours after the onset of symptoms using the Nitro B.T. method. Also, It coincided with the findings of Andersen and Hensen[13,14] who detected localized subendocardial infarctions of 1 hour duration. They however reason that subendocardial infarctions presumably had a symptoms free period. It was also the hypothesis of Gregersen[15] that all infarcts start in the subendocardial zone.

We might deduce that perhaps the subject had survived for a longer period of time a transmural infarction might have been demonstrated due to the loss of enzyme activity. This discrepancy in the time required for demonstration of loss of enzyme activity could also be due to unreliability of fixing the actual event of infarction to coincide with onset of pain or any other clinical symptoms.

The age of an infarct is notoriously difficult to establish in the human, as the onset of clinical symptoms, however dramatically abrupt, are often much later than the onset of the pathological lesion precipitated by a coronary occlusion. In animal experiments, a coronary vessel can be ligated at zero time and serial sacrifices made at different intervals to gain an accurate estimate of the age of the infarct. In the human, the time of chest pain and shock cannot be used in a similar fashion. When a victim of coronary disease dies say, eight hours after the onset of acute symptoms, though one might expect an early infarct to be visible on histological or histochemical examination, not frequently a well-demarcated yellow or tigroid area of necrosis is present; which must be several days old.

Conclusion:

This study shows that the Maximum number of early myocardial infarction (less than 6 hour of duration) can be detected by TTC method. The technique of this procedure is quite simple and easy to perform. The reagents required are not out of the reach (economically) for medium sized laboratories. As regards interpretation of results, the TTC macro-method did not pose any problems. While carrying out this type of study to minimize false results, selection of proper sample type is very important. An attempt should be made to establish TTC macro method at all the hospitals including primary centers where the postmortems are conducted.

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