Open Access

ORIGINAL ARTICLE

Enhancement of myocardial recovery with terminal ‘hot shot’ cardioplegia

Shahbaz Ahmad Khilji[1], Muhammad Fahad Ghaffar [2]

Department of Cardiac Surgery, Faisalabad Institute of Cardiology, Faisalabad, Pakistan.[1,2]

ABSTRACT

Background: Terminal ‘hotshot’ (THS) cardioplegia is a technique that might contribute into myocardial protection in patients undergoing cardiac surgery.

Objective: To determine the effect of THS cardioplegia administration in patients undergoing coronary artery bypass grafting (CABG).

Methods: A randomized control trial (ACTRN12624001225505) was conducted from 20th December 2023 to 10th February 2024 involving 60 patients admitted from the outpatient department for elective CABG at Faisalabad Institute of Cardiology, Faisalabad, Pakistan. It was a single-blinded study and the patients were randomized into two equal groups, group A (control group) and group B (experiment group). During the surgery performed utilizing a Cardiopulmonary Bypass (CPB) machine, both groups received cold blood cardioplegia to arrest the heart, providing a quiescent field at the start of the grafting process in CABG. In group B patients, 100 ml of THS volume was given to each of the grafted territories via saphenous vein grafts and the rest of the volume from the total of 500 mL was given in the aortic root via the antegrade cardioplegia cannula for maximum myocardial distribution. The levels of Troponin I (TnI) and Creatinine Kinase MB (CK MB) were measured in the blood one hour after regaining sinus rhythm and on 5th postoperative day (POD). Durations of postoperative Inotrope use, intubation, aortic cross-clamp, CPB time, time to regain sinus rhythm, and ICU stay were recorded in both the groups. Data was entered and analyzed using SPSS version 25. A p-value ≤ 0.05 was considered statistically significant.

Results: Mean age of the participants was 58.9±7.36 years. The levels of TnI were lower in group B than in group A one hour after regaining sinus rhythm (0.20±0.076 ng/ml and 0.31±0.058 ng/ml respectively, p-value <0.001) as well as on the 5th POD (0.15±0.663 ng/ml and 0.26±0.051 ng/ml respectively, p-values<0.001). CK MB levels were also lower in group B than in group A one hour after regaining sinus rhythm (48.4±25.13 IU/L and 70.5±29.00 IU/L respectively, p-value <0.003) as well as on the 5th POD (39.2±19.44 IU/L and 71.0±37.08 IU/L respectively, p-value<0.001). Inotropic support was used for a shorter time in group B than in group A (5.9±1.99 hours and 7.0±1.87 hours respectively, p-value=0.031). There were no significant differences in the durations of intubation, ICU stay, cross-clamp time, CPB time, and the time to regain sinus rhythm between the two groups (p-value>0.05).

Conclusion: ‘Hot shot’ cardioplegia decreases the release of TnI from the myocardial cells post-cardiac surgery. The inotropic support time is also shortened when THS cardioplegia is used.

Key Words: Cardioplegia, Terminal hot shot, Cardiac surgery, Myocardial recovery

Doi: https://doi.org/10.53685/jshmdc.v5i2.229

Corresponding Author:
Muhammad Fahad Ghaffar
Postgraduate Trainee
Department of Cardiac Surgery, Faisalabad Institute of Cardiology, Faisalabad, Pakistan
Email address: fahad.mfg22@gmail.com
Received: 16.06.24, 1st Revision: 12.09.24,
2st Revision: 4.11.24, Accepted 15.11.24

……
……

How to cite this article: Khilji SA and Ghaffar MF. Enhancement of myocardial recovery with terminal ‘hot shot’ cardioplegia. J Shalamar Med Dent Coll. 2024; 5(2):89-94. doi: https://doi.org/10.53685/jshmdcv5i2.229

INTRODUCTION

Myocardial protection is the cornerstone of cardiac surgery.[1] One of the most common causes of low cardiac output syndrome (LCOS) post-cardiac surgery is inadequate myocardial protection. Since the advent of cardiac surgery ventricular fibrillation with intermittent aortic cross-clamping, deep systemic hypothermia and cardioplegia with various ionic compositions and temperatures have assisted in achieving this goal. In the past, a frequently applied technique to achieve this was the use of terminal 'hot shot' (THS) cardioplegia which was administered to

enhance myocardial recovery. THS includes the components that are responsible for stabilizing the myocardial cell membrane and decreasing metabolic content intracellularly. It can be given via saphenous vein grafts, the aortic root, or retrograde through the coronary sinus. This technique has shown promising results in myocardial recovery with the earlier return of spontaneous cardiac activity after aortic cross-clamp removal.[2] The effects of this strategy have been tested in animal subjects and shown to have superior myocardial recovery and less LCOS postoperatively.[3] Among the patients receiving a 'hot shot', there were significantly higher ATP levels and reduced lactate levels in myocardial biopsies post-reperfusion, consistent with improved myocardial recovery.[4]

The use of THS on cardiopulmonary bypass grafting (CABG) patients has been known to decrease the need for defibrillating the heart while weaning from cardiopulmonary bypass (CPB) emphasizing global myocardial recovery.[5] Most of the research done internationally was retrospective,[2,5] had a smaller sample size,[3,6] used either warm blood cardioplegia,[7,8,9] or crystalloid cardioplegia,[3] or measured variables over a short period of time.[2] In our country, there has been no study on cardiac surgery setups using THS in the last 10 years. The objective of our study was to determine the effect of Terminal 'hot shot' cardioplegia administration in patients undergoing CABG.

METHODS

A randomized controlled trial was conducted at the Cardiac Surgery Department of Faisalabad Institute of Cardiology, Faisalabad, Pakistan from 20th December 2023 to 10th February 2024. It was a single-blinded study; written informed consent was taken and 60 patients were enrolled in the study. Patients were randomly allocated into two equal groups by computer-generated software (research randomizer), group A was the control group and group B was the experimental group.[2]

Patients above 18 years of age, males and females, with body mass index (BMI) of 18.5 to 29.9; and admitted from the outpatient department for the elective CABG were included in the study. Patients with left ventricular ejection fraction (LVEF) of less than 45% and left main stem stenosis were excluded from this study.

The operational definition of cold blood cardioplegia is the volume of blood at 4° C enriched with potassium to stop the heart temporarily in the patients during cardiac surgery while they are undergoing the cardiopulmonary bypass (CBP). Terminal 'hot shot' cardioplegia is an enhanced cell membrane stabilizing mixture of blood containing 20% Mannitol, 60 mg 2% Xylocaine, 500 mg Magnesium Sulphate and 8.4% Sodium Bicarbonate at 37° C. It is administered prior to the removal of cross-clamp previously placed on the aorta at the start of grafting.

Group A: Control group; patients undergoing CABG and no THS cardioplegia intervention was administered.

Group B: Experiment group; patients undergoing CABG and THS cardioplegia intervention was administered.

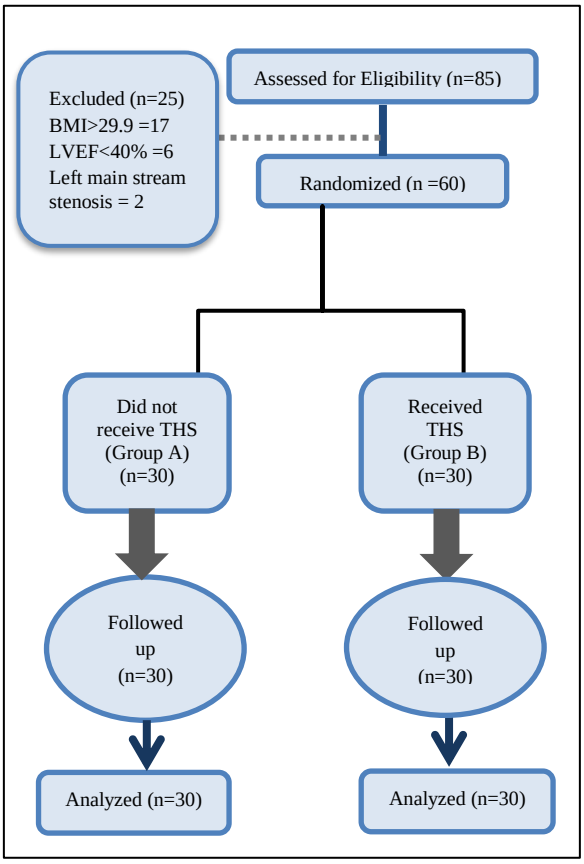

Figure 1: Flowchart of patient recruitment, allocation, intervention and analysis

After anesthesia induction and aseptic measures, median sternotomy and pericardiotomy were done. The left internal mammary artery (LIMA) and long saphenous vein were harvested. The patient was then

heparinized, and aortic cannulation (distal ascending aorta) and two staged venous cannulations (right atrial appendage) were done. CBP was initiated, aortic cross-clamp was applied, followed by administration of cold blood cardioplegia in both groups to arrest the heart. The temperature was reduced to 32° C. Distal coronary anastomosis was secured and rewarming started. In group B patients, 100 ml of THS volume was given to each of the grafted territories via saphenous vein grafts at this point. The rest of the 500 mL of THS volume was given in the aortic root via the antegrade cardioplegia cannula for maximum myocardial distribution.

During administration, the delivery of THS was visually confirmed by the operating surgeon, assistant surgeon, and the perfusionist, for its rundown in the coronaries. After THS was given, the aortic cross-clamp was removed, keeping low flows in CPB. The top ends of grafts were anastomosed to the aorta. Time taken by the heart to regain sinus rhythm after cross-clamp removal was recorded. The patient was weaned off from CPB. Blood samples for TnI and CK MB were taken at two points; 01 hour after regaining sinus rhythm and at the 5th postoperative day (POD). CPB time and the time for the return of sinus rhythm were measured intraoperatively. Postoperative durations of intubation, inotropic support, and ICU stay were also recorded.

Ethical Approval

The ethical approval of the study was obtained from the Ethical Review Board of Faisalabad Institute of Cardiology, Faisalabad, Pakistan (letter number 46-2023/DME/FIC/FSD). This trial was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) number ACTRN12624001225505.

Statistical Analysis

The data analysis was conducted using SPSS version 25. Continuous variables were presented as mean ± standard deviation (SD), while categorical data were reported as frequencies and percentages. An independent t-test was used to compare the quantitative data in groups. A p-value of ≤ 0.05 was considered significant.

RESULTS

The mean age of the study participants was 58.9±7.36 years, comprising of 58% males and 42% females. The mean BMI of the participants was 24.7±1.80 kg/m^2. Diabetes was the most common co-morbidity observed, affecting more than half of the patients, and hypertension was in 42% of the patients. LVEF values varied, with the majority having an LVEF of 60%, while smaller proportions had values ranging from 45% to 55%, indicating potential an early-stage cardiac compromise (Table 1).

Table 1: Baseline characteristics of the whole study population

Variables	n = 60 n(%)
Hypertensive	25(42)
Diabetic	32(53)
COPD	21(35)
LVEF	
45%	10(17)
50%	6(10)
55%	9(15)
60%	35(58)

COPD: Chronic obstructive pulmonary disease, LVEF: Left Ventricle ejection fraction %

Table 2 displays the baseline characteristics of the patients in groups A and B. There were no significant differences in gender, hypertension, diabetes mellitus, COPD, and LVEF between the two groups.

Table 2: Baseline characteristics of the patients in the control and the experimental groups

Variables	Group A (n=30) n (%)	Group B (n=30) n (%)	χ^2	p-value
Gender				
Male	20 (67)	15 (50)		
Female	10 (33)	15 (50)	1.71	0.19[a]
Hypertension				
Yes	9 (30)	16 (53)		
No	21 (70)	14 (47)	3.36	0.06 [a]
Diabetes mellitus				
Yes	19 (63)	13 (43)		
No	11(37)	17(57)	2.41	0.12 [a]
COPD				
Yes	10 (33)	11 (37)		
No	20(67)	19 (63)	0.07	0.79 [a]
LVEF				
45%	5 (17)	5 (17)		
50%	3 (10)	3 (10)		
55%	6 (20)	3 (10)		
60%	16 (53)	19 (63)	NA	0.73[b]

[a]Chi Square test was applied. [b]Fisher's Exact test applied. p-vlaue≤0.05 was considered statistically significant. COPD: Chronic obstructive pulmonary disease, LVEF: Left Ventricle ejection fraction %, Group A: Control group, Group B: Experiment group,

The intraoperative and immediate post-operative variables were compared between groups A and B. No significant differences were found in cross-clamp

time, CPB time, or the time to resume sinus rhythm between the two groups (p-value>0.05). However, Troponin I and CK-MB levels, assessed one hour after regaining sinus rhythm, were lower in group B than in group A (p-value<0.05). Additionally, group A required longer inotropic support post-operatively than group B (p-value=0.031). No significant differences were found in intubation duration and ICU stay between the two groups (p-value>0.05). The levels of TnI and CK MB levels on the 5th post-operative day were lower in group B as compared to group A (p-value<0.001) (Table 3).

Table 3: Comparison of intraoperative and postoperative variables between the two groups

Variable	Group A Mean±SD	Group B Mean±SD	p-value
Cross clamp time (min)	58.8±9.20	59.6±8.25	0.724
CPB time (min)	86.8±12.65	87.9±12.09	0.732
Time to regain SR (s)	137.2±22.37	140.2±29.05	0.656
Troponin I (ng/ml)			
1 hr after SR	0.31±0.058	0.20±0.076	<0.001*
CK MB (IU/L)			
1 hr after SR	70.5±29.00	48.4±25.13	0.003*
Intubation duration (hr)	4.5±1.59	4.2±1.29	0.376
ICU stay (days)	2.7±0.58	2.6±0.62	0.521
Inotropic support (hr)	7.0±1.87	5.9±1.99	0.031*
5th POD Troponin I (ng/ml)	0.26±0.051	0.15±0.663	<0.001*
5th POD CK MB (IU/L)	71.0±37.08	39.2±19.44	<0.001*

*Independent samples t-test was applied. *p-vlaue≤0.05 was considered statistically significant. Group A: Control group, Group B: Experiment group. CPB=cardiopulmonary bypass, SR=sinus rhythm, POD=post-operative day*

DISCUSSION

In this randomized trial, TnI and CK MB levels were compared between control patients and patients who received THS cardioplegia during CABG and the levels were significantly lower in the experiment group than the control group, both in the initial intraoperative period as well as on the fifth postoperative day. This indicates a lesser cardiac enzyme response in the THS-treated patients during the early recovery period post-CPB, suggesting an association between THS administration and decreased myocardial stress. We also found that the inotropic support time was shorter in the experiment group than in the control group.

Some surgeons have practiced the use of THS during CPB, yet it is not universally adopted due to the additional time it requires, which can increase aortic cross-clamp and CPB durations. This is significant as prolonged CPB time has been directly linked to patient mortality, as reported in a study.[10] Cardiac surgeons are continually striving to minimize these time durations to reduce potential adverse effects. In the present study, we aimed to deliver THS efficiently, ensuring effective myocardial distribution while minimizing the increases in cross-clamp and CPB time, which could be especially beneficial in procedures requiring extended CPB.[11] Enhanced myocardial recovery using THS may also mitigate risks of acute kidney injury, as prolonged CPB has been associated with acute kidney injury in extensive thoracic aorta surgeries, and an improved myocardial protection may positively impact renal function.[12]

The main finding of this study was the lower TnI levels in the group in which THS cardioplegia was used than the control group. TnI is widely recognized as a sensitive marker of intraoperative myocardial injury. The TnI increases in all patients after cardiac surgery. This fact shows the inevitable myocardial damage caused by myocardial arrest. Nanni et al showed that a rise in TnI post-surgery is directly related to more than 10% decrease in LVEF.[13] Any maneuver which keeps a low TnI level postoperatively should be employed. Using THS cardioplegia, we can keep the postoperative TnI levels under control, not just in the immediate postoperative period but also over a longer period of five days. Both these values were significantly lower in the experiment group than the control group. Similar trend in TnI was observed in a previous study on CABG patients by Ji et al. where its levels were found to be lower in the group subjected to warm induction and reperfusion blood cardioplegia than in the group of simple warm induction with no reperfusion.[9] They used warm blood cardioplegia for induction unlike our utilization of the cold blood cardioplegia. The warm and cold blood cardioplegia, both have shown low incidences of death, perioperative myocardial infarction, and the need of intra-aortic balloon pump support. Still, warm blood cardioplegia (37°C) protects from the deleterious effects of the hypothermic ischemia and minimizes reperfusion injury which could contribute to a decrease in TnI.[14,15,16] In another study, the THS temperature was 20°C initially and subsequently rewarmed to 35°C at the end of reperfusion which lasted almost 06 minutes.[17] THS cardioplegia administration via grafts saphenous vein was not performed, however, THS used in the present study was at 37°C constant temperature and was infused in

05 minutes via both the grafted saphenous veins and aortic root.

Karaarslan et al. in his retrospective observational study performed on CABG patients, showed a decrease in Troponin levels in the group receiving THS although not a statistically significant result.[2] Similar results were obtained by Caputo et al. in which 35 patients undergoing CABG with a LVEF >50% were randomized into two groups, a control and a THS group, and their cumulative mean TnI release over a period of 48 hours was calculated. No significant difference was found between the two groups. They only infused THS in the aortic root and no volume was administered via the saphenous vein grafts.[6]

The studies done on valvular cardiac surgery were even less promising in terms of TnI release when compared between a THS and a non THS groups.[8] In one of the studies, 70 patients who underwent first time elective valvular surgery had their total amount of cardiac troponin I measured. There was no significant difference in TnI levels between the group receiving THS and the control group.[17] In another study, the levels of TnI at 6 hours postoperatively were 0.57 ng/ml in the THS group and 0.74 ng/ml in the non-THS group with no significant difference.[18] The role of THS in congenital cardiac surgery in patients who underwent complex repair in terms of TnI release has also not been significant.[19,20] In light of the above, the effect of THS is more profound in ischemic cardiac surgeries.

In this study, CK-MB levels were found to be lower in the experiment group compared to the control group at both the first and fifth postoperative days. Similar trends were observed in a meta-analysis by Zhou et al., which highlighted significantly lower CK-MB levels in THS groups post-operatively.[21] Though CK-MB is less specific to myocardial injury, its levels are considered an adjunct marker in cardiac recovery.

In this study, CK-MB levels were found to be lower in the experiment group than in the control group on both the first and fifth postoperative days. Similar trends were observed in a meta-analysis by Zhou et al., which highlighted significantly lower CK-MB levels in THS groups post-operatively.[21] Though CK-MB is less specific to myocardial injury, its levels are considered an adjunct marker in cardiac recovery.

Our study showed no significant difference in the time required for the spontaneous return of cardiac activity between the experiment group and the control group. Another study showed that it may be positively influenced by THS.[2] In a study done in Pakistan in 2012, spontaneous return of cardiac activity, being the primary variable, was seen in less time in the THS group as compared to the non-THS group. [22]

The cumulative data todate underscores that slow myocardial recovery following ischemic injury contributes significantly to morbidity and mortality. THS provides a straightforward, accessible means of enhancing myocardial recovery post-ischemic insult, especially in ischemic cardiac surgeries, and shows promise in preserving both myocardial and renal function by limiting cardiac enzyme release and enhancing recovery markers.

CONCLUSION

Terminal 'hot shot' cardioplegia administration decreases Troponin I levels and enhances myocardial recovery. It assists in reducing the duration of inotropic support required postoperatively. With further workup, it can be one of the fundamental strategies in myocardial tissue preservation during coronary artery bypass grafting.

Limitations of the study

The sample size in the present study was small. Multiport cardioplegia administration was not available, due to which all territories grafted did not receive hot shots simultaneously, leading to prolonged aortic cross-clamp and CPB time.

Future Recommendations

Studies with large sample size over a prolonged period and with myocardial biopsies taken are needed to determine further uses and implications of THS.

REFERENCES

1. Beyersdorf F. Intraoperatoive myocardial protection: still the cornerstone of cardiac surgery. Eur J Cardiothorac Surg. 2022 ;61(5):1133-1134. doi:10.1093/ejcts/ezab510
2. Karaarslan K, Abud B. Effects of del Nido and terminal warm blood cardioplegia on myocardial protection and rhythm in isolated CABG patients. Heart Surg Forum. 2021;24(5):E808-e813. doi:10.1532/hsf.4103
3. Nakao M, Morita K, Shinohara G, Saito S, Kunihara T. Superior restoration of left ventricular performance after prolonged single-dose del Nido cardioplegia in conjunction with terminal warm blood cardioplegic reperfusion. J of Thorac Cardiovasc Surg. 2022;164(4):e143-e153. doi: 10.1016/j.jtcvs.2020.11.152

4. Volpi S, Ali JM, De Silva R. Does the use of a hot-shot lead to improved outcomes following adult cardiac surgery? Interact Cardiovasc Thorac Surg. 2019;28(3):473-477. doi: 10.1093/icvts/ivy266
5. Mirmohammadsadeghi P, Mirmohammadsadeghi M. Effects of single antegrade hot shot in comparison with no hot shot administration during coronary artery bypass grafting. ARYA Atheroscler. 2015;11(3):186-190.
6. Caputo M, Dihmis WC, Bryan AJ, Suleiman MS, Angelini GD. Warm blood hyperkalaemic reperfusion ('hot shot') prevents myocardial substrate derangement in patients undergoing coronary artery bypass surgery. Eur J Cardiothorac Surg. 1998;13(5):559-564. doi: 10.1016/s1010 7940(98)00056-
7. Teoh KH, Christakis GT, Weisel RD, Fremes SE, Mickle DA, Romaschin AD, et al. Accelerated myocardial metabolic recovery with terminal warm blood cardioplegia. J Thorac Cardiovasc Surg. 1986;91(6):888-895.
8. Ascione R, Suleiman SM, Angelini GD. Retrograde hot-shot cardioplegia in patients with left ventricular hypertrophy undergoing aortic valve replacement. Ann Thorac Surg. 2008;85(2):454. doi: 10.1016/j.athoracsur.2007.08.039
9. Ji B, Liu M, Lu F, Liu J, Wang G, Feng Z, et al. Warm induction cardioplegia and reperfusion dose influence the occurrence of the post CABG TnI level. Interact Cardiovasc Thorac Surg. 2006;5(1):67-70. doi: 10.1510/icvts.2005.1157 41
10. Madhavan S, Chan SP, Tan WC, Eng J, Li B, Luo HD, et al. Cardiopulmonary bypass time: every minute counts. J Cardiovasc Surg. 2018;59(2):274-281. doi: 10.23736/S0021-9509.17.09864-0
11. Xu S, Liu J, Li L, Wu Z, Li J, Liu Y, et al. Cardiopulmonary bypass time is an independent risk factor for acute kidney injury in emergent thoracic aortic surgery: a retrospective cohort study. J Cardiothorac Surg. 2019;14(1):90. doi: 10. 1186/s13019-019-0907-x
12. Jin G, Seong SW, Kim MJ, Ahn KT, Jin SA, Hahn JY, et al. Prevalence and Prognostic Implications of Worsening Renal Function After Acute Myocardial Infarction. Am J Cardiol. 2023;200:40-46. doi: 10.1016/j.amjcard.2023.05.011
13. Nanni S, Garofalo M, Schinzari M, Nardi E, Semprini F, Battistini P, et al. Prognostic value of high-sensitivity cardiac troponin I early after coronary artery bypass graft surgery. J Cardiothorac Surg. 2022;17(1):280. doi: 10.1186/ s13019-022-02027-x
14. Mauney MC, Kron IL. The physiologic basis of warm cardioplegia. Ann Thorac Surg. 1995;60(3):819-823. doi: 10.1016/0003-4975(95)00422-H
15. James TM, Nores M, Rousou JA, Lin N, Stamou SC. Warm Blood Cardioplegia for Myocardial protection: Concepts and controversies. Tex Heart Inst J. 2020;47(2):108-116. doi: 10. 14503/THIJ-18-6909
16. Falcoz PE, Kaili D, Chocron S, Stoica L, Toubin G, Puyraveau M, et al. Blood warm reperfusion: a necessary adjunct to heart-valve surgery in low-risk patients? J Cardiovasc Surg. 2005;46(6):577-581.
17. Kot TKM, Chan JSK, Froghi S, Lau DHH, Morgan K, Magni F, et al. Warm versus cold cardioplegia in cardiac surgery: A meta-analysis with trial sequential analysis. J Thorac Cardiovasc Surg. 2021;6:161-190. doi: 10.1016/j. xjon.2021.03. 011
18. Rergkliang C, Chetpaophan A, Chittithavorn V, Vasinanukorn P, Chowchuvech V. Terminal warm blood cardioplegia in mitral valve replacement: prospective study. Asian Cardiovasc Thorac Ann. 2006;14(2):134-138. doi: 10. 1177/021849230601400211
19. Busro PW, Romolo H, Sastroasmoro S, Rachmat J, Sadikin M, Santoso A, et al. Role of terminal warm blood cardioplegia in complex congenital heart surgery. Asian Cardiovasc Thorac Ann. 2018;26(3):196-202. doi: 10.1177/ 0218492318759105
20. Modi P, Suleiman MS, Reeves B, Pawade A, Parry AJ, Angelini GD, et al. Myocardial metabolic changes during pediatric cardiac surgery: a randomized study of 3 cardioplegic techniques. J Thorac Cardiovasc Surg. 2004;128(1):67-75. doi: 10.1016/j.jtcvs.2003.11.071
21. Zhou K, Zhang X, Li D, Song G. Myocardial protection with different cardioplegia in adult cardiac surgery: A network meta-analysis. Heart Lung Circ. 2022;31(3):420-429. doi: 10.1016/j.hlc.2021.09.004
22. Janjua AM, Iqbal MA, Ali N, Rashid A, Bakhsh M, Siddiq R. Effects of hot shot (non cardioplegic blood based) on cardiac contractility and rhythm as parameters of myocardial protection in CABG surgery. Pak Armed Forces Med J. 2012;62(1):20-24.

AUTHORS' CONTRIBUTION:

SAK: Conceptualizing, data analysis and interpretation, critical review, approval of the final version to be published

MFG: Data collection, manuscript drafting, critical review, approval of the final version to be published

All Authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved

CONFLICT OF INTEREST:

All authors declared no conflict of interest.

GRANT SUPPORT AND FINANCIAL DISCLOSURE:

No specific grant was taken for this research from any funding agency in the public, commercial or not-for-profit sectors.

DATA SHARING STATEMENT:

The data are available from the corresponding author upon reasonable request.

……