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ORIGINAL ARTICLE

Prophylactic treatment with alkaline phosphatase in cardiac surgery induces endogenous alkaline phosphatase release

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ABSTRACT

Introduction: *Laboratory and clinical data have implicated endotoxin as an important factor in the inflammatory response to cardiopulmonary bypass. We assessed the effects of the administration of bovine intestinal alkaline phosphatase (bIAP), an endotoxin detoxifier, on alkaline phosphatase levels in patients undergoing coronary artery bypass grafting.*
Methods: *A total of 63 patients undergoing coronary artery bypass grafting were enrolled and prospectively randomized. Bovine intestinal alkaline phosphatase (n=32) or placebo (n=31) was administered as an intravenous bolus followed by continuous infusion for 36 hours. The primary endpoint was to evaluate alkaline phosphatase levels in both groups and to find out if administration of bIAP to patients undergoing CABG would lead to endogenous alkaline phosphatase release.*
Results: *No significant adverse effects were identified in either group. In all the 32 patients of the bIAP-treated group, we found an initial rise of plasma alkaline phosphatase levels due to bolus administration (464.27±176.17 IU/L). A significant increase of plasma alkaline phosphatase at 4-6 hours postoperatively was observed (354.97±95.00 IU/L) as well. Using LHA inhibition, it was shown that this second peak was caused by the generation of Tissue Non Specific Alkaline Phosphatase (TNSALP-type alkaline phosphatase).*
Conclusions: *Intravenous bolus administration plus 8 hours continuous infusion of alkaline phosphatase in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass results in endogenous alkaline phosphatase release. This endogenous alkaline phosphatase may play a role in the immune defense system.*

KEY WORDS: *Alkaline phosphatase, Cardiopulmonary bypass, Cardiac surgery*

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INTRODUCTION

Alkaline phosphatase is an endogenous ecto-enzyme that is ubiquitous in the human body. This ectophosphatase is widely expressed in many organs that are exposed directly or indirectly to the external environment, like the gastrointestinal tract and the lungs. A physiological role for alkaline phosphatase was proposed in 1997 by Poelstra et al (1).

Alkaline phosphatase dephosphorylates and thereby detoxifies not only endotoxins (lipopolysaccharides) but also extracellular nucleotides (2, 3). Alkaline phosphatase converts these nucleotides into non-inflammatory nucleosines (4). Both endotoxins and nucleotides are potent inflammatory triggers and are sensed as 'stranger' or 'danger' signals to the innate immune system; consequently, local and systemic inflammatory responses (SIRS) may result from the exposure to these pro-inflammatory signals (5).

During cardiopulmonary bypass (CPB), hypoperfusion of the gut may result in a loss of barrier function and, as a consequence, bacterial endotoxins, normally confined to the lumen of the intestine by a barrier of endovascular cells, may enter the systemic circulation (6, 7). The amount of endotoxin release into the circulation seems to be related to the duration of cross clamping time and CPB time. In this regard, endotoxin release has been recognized as an important factor in the inflammatory response following CPB (8).

In previous animal studies, promising therapeutic effects have been shown in reducing the inflammatory response with the use of intravenous alkaline phosphatase (9-11). In a clinical study in severe sepsis patients, continuous infusion of calf-intestinal alkaline phosphatase significantly improved renal function (12, 13). It is known that the pro-and anti-inflammatory response can be manipulated by positive feedback mechanisms. However, it is not known whether alkaline phosphatase infusion can induce such a positive feedback. In an earlier report, we studied the possible beneficial effect of bovine intestinal alkaline phosphatase (bIAP) on inflammatory parameters post-CPB in a randomized, double blind, placebo-controlled study with bIAP in patients undergoing elective coronary artery bypass grafting (CABG) with the use of CPB (the APPIRED study) (14). In that phase of the study, we investigated the effect of bIAP on inflammatory parameters after CPB and the possible clinical effects of bIAP on the prevention of systemic inflammatory response syndrome (SIRS). In the present sub-study, we investigated the levels of alkaline phosphatase in patients undergoing CABG with CPB, treated with either placebo or bIAP. We hypothesized that administration of bIAP would increase endogenous alkaline phosphatase release in patients undergoing CABG with the use of CPB.

MATERIALS AND METHODS

In a double blind, placebo-controlled study, 63 patients undergoing elective CABG with the use of CPB were randomized to receive either bIAP (n=32) or matching placebo (n=31). The study was approved by the Institutional Review Board. An informed consent was obtained from each participant. The study drug bIAP was manufactured by Biozyme ltd (Bleanavon, Wales, UK) and Alloksys Life Sciences B.V. (Bunnik, The Netherlands). The placebo also consisted of 1 mL sterile aqueous solution for infusion in an aqueous buffer containing 20 mM Tris-HCl, 5 mM Magnesium Chloride, 0.1 mM Zinc Chloride, pH 7.3, with 25% glycerol containing no bIAP. The study drug (bIAP) or matching placebo was administered as an intravenous bolus of 1000 International Units (IU), just prior to induction of anesthesia, directly followed by intravenous continuous infusion of 5.6 units per kilogram per hour at a flow rate of 4 mL per hour for 36 hours in order to maintain supranormal levels of alkaline phosphatase in blood. A phase I bIAP study demonstrated that 72-hour continuous infusions of up to a total of 16,000 IU to 48,000 IU (at 80 kg bodyweight) of bIAP was safe and well tolerated. No immune incompatibility was found, as evidenced by lack of induction of specific antibodies to bIAP over a period of 90 days after administration. No drug-related adverse events were reported (15).

The priming fluid of the CPB consisted of 800 mL NaCl 0.9%, 500 mL Voluven® (Fresenius Kabi, Zeist, the Netherlands), 200 mL Mannitol 20% (Baxter Health Care, Utrecht, the Netherlands), 200 mL Aprotinin 1000 KIU/mL, 25 mL NaHCO3 8.4% and Heparin 7500 IU.

Blood samples (hematological parameters, clinical chemistry, cytokines including IL-6, IL-8 and TNFα, and anti-endotoxin antibody) were collected at several time points before, during and after surgery (24 and 0.25 hr before induction of anesthesia and 0.25 hr, 1.5 hr, 3 hr, 4 hr, 12 hr, 24 hr, 35, 37 and 96 hr after induction of surgery, pointed out as visit numbers 1-11). Furthermore, clinical parameters like length of ICU stay, duration of ventilation, and length of hospital stay were recorded. An extended description of the materials and methods has been described earlier (14). The primary endpoint of the present sub-study was to evaluate alkaline phosphatase levels in both groups and to find out if administration of bIAP to patients undergoing CABG would lead to endogenous alkaline phosphatase release.

Alkaline phosphatase measurement

Alkaline phosphatase was measured using a PNPP (p-nitrophenol phosphate) kinetic assay (16). Samples were defrosted and warmed gradually to 21 degrees Celsius. A total of 200 µL of a serum sample was mixed with 1 mL of PNPP-substrate (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and $MgCl_2$ (final concentration 2 mM) in a Tris-glycerin buffer at pH 9.6. Samples were measured kinetically at 405 nm on a Bio-Rad (Hercules, CA, USA) Smartspec photospectrometer for 60 seconds with intervals of 20 seconds.

L-homo arginine (LHA) inhibition

L-homo arginine (LHA) (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was used as a tissue, non-specific, alkaline phosphatase (TNSALP) inhibitor, to investigate the origin of the endogenous alkaline phosphatase. LHA is supposed to have minimal influence on bIAP, but inhibits tissue non-specific alkaline phosphatase activity (17). BIAP was diluted to a concentration of 0.08 U/L and 100 µL was added to a cuvette; 5 mM LHA (final concentration) was added and this was gently mixed and incubated at

TABLE I - BASELINE CHARACTERISTICS

Variable	bIAP (n=32)	placebo (n=31)	p
Preoperative data			
Age (y),	71.4±4.2	70.2±6.8	0.495
Male, n	27 (84.4%)	28 (90.3%)	0.478
BMI (kg/m²)	27.4±3.5	25.7±2.7	0.037
Euroscore (additive)	3.63±1.24	3.68±1.42	0.682
EF <30%, n	3 (9.4%)	6 (19.4%)	0.535
COPD, n	1 (3.1%)	4 (12.9%)	0.151
Diabetes, n	6 (18.8%)	3 (9.7%)	0.304
Preop serum creatinin (µmol/L)	90.6±20.1	95.5±17.5	0.153
Operative data			
Number of grafts	3.8±1.1	3.5±0.9	0.078
Total duration of surgery (hours)	2.95±1.08	2.76±0.58	0.874
CPB duration (min)	72.2±41.2	61.5±22.3	0.257
Cross clamp duration (min)	49.4±27.3	44.7±16.8	0.710
Use of cell saver, n	12 (37.5%)	15 (48.4%)	0.374
Warm blood cardioplegia, n	23 (71.9%)	24 (77.4%)	0.613
St. Thomas cold crystalloid cardioplegia, n	9 (28.1)	7 (22.6)	0.613
Concomitant PVISO	1 (3.1%)	1 (3.2%)	ns
Postoperative data			
Intensive care length of stay (hours)	18.3±1.5	16.5±1.8	0.146
Hospital length of stay (days)	6.1±2.4	6.2±2.8	0.847
Hospital readmission, n	0 (0%)	2 (6.4%)	0.144
30 day mortality, n	1 (3.1%)	1 (3.2%)	ns
Postoperative atrial fibrillation, n	9 (28.1%)	12 (38.7%)	0.373
Postoperative infections, n	3 (9.6%)	3 (9.6%)	ns
New-onset stroke, n	0 (0%)	1 (3.2%)	0.306

Data are expressed as mean±SD or numbers (%) unless otherwise mentioned.
BMI = body mass index; COPD = chronic obstructive pulmonary disease; CPB = cardiopulmonary bypass; EF = ejection fraction; PVISO = pulmonary vein isolation.

room temperature for 5 minutes. Next a PNPP kinetic assay was carried out similarly as described above, but with measurements for 180 seconds with intervals of 20 seconds. For pharmacokinetic purposes, alkaline phosphatase levels in human blood plasma samples were measured at the Catharina Hospital Laboratory by routine clinical chemistry methods (Cobas-Bio centrifugal analyzer; Roche Diagnostics, Basel, Switzerland).

Statistical analysis

When applicable, evaluation was performed with help of the SAS System (Software Release 9.13). Data were checked for completeness and a second plausibility check was performed. The Wilcoxon signed rank test was used to compare continuous variables; the Pearson's chi-square test was used to investigate the frequency (percentage) to parameters; and a probability of $p < 0.05$ was considered to be statistically significant.

RESULTS

A total of 63 patients (bIAP n=32, placebo n=31) was enrolled in this study. No significant safety concerns were identified. Baseline characteristics are listed in Table I. Beside a significantly higher BMI in the bIAP-treated group, no statistical significant differences in demographic data were observed.

Hospital mortality included one patient in each group. The patient in the BIAP group was a 70-year-old male with a normal LVEF. Intraoperatively, after release of the cross clamp, he developed resistant ventricular fibrillation. After complete revision of all bypass grafts, he was weaned from the ECC and transported to the ICU with inotropic support. However, a diagnosis of perioperative myocardial infarction was made. On the 7th postoperative day, he suddenly had a cardiac arrest without picture of tamponade on echocardiography. The patient in the placebo group had a Euroscore of 6. He was an 83-year-old male with a normal LVEF. He developed perioperative myocardial ischemia. The coronary system was described as "sick" and "small" by the operating surgeon who had to insert an intra-aortic balloon pump in trying to wean the patient from the ECC. The patient developed postoperative cardiogenic shock and died of respiratory insufficiency on

the 11th postoperative day.

An evident inflammatory response was observed in only 5 patients of the placebo group. In these 5 patients, we observed a fulminant TNFα response (mean peak level 108.1 pg/mL) at 3 to 4 hours after the start of surgery. This TNFα response was followed by an increase in plasma levels of IL-6 and IL-8 (mean peak levels of 682.6 pg/mL and 641.9 pg/mL, respectively). A TNFα response of this sort was not observed in the bIAP treated group ($p < 0.02$). The overall inflammatory response as deduced from cytokine levels, C reactive protein (CRP), AST, and ALT was low in both the bIAP-treated group and the placebo group. No significant differences in peri-operative complications were found between the groups.

Postoperative plasma Alkaline Phosphatase levels

Preoperative levels of alkaline phosphatase were 70.03±17.12 IU/L in the bIAP treated group, and 70.50±15.63 IU/L in the placebo treated group (p=ns). In all 31 patients of the placebo-treated group, we found a reduction of plasma alkaline phosphatase levels within 2 hours after start of surgery (34.89±9.59 IU/L). This reduction in plasma alkaline phosphatase levels was followed by normalization of this level after 24 hours.

In all the 32 patients of the bIAP-treated group we found an initial rise of plasma alkaline phosphatase levels due to bolus administration (464.27±176.17 IU/L). Next to the initial rise, a significant increase of plasma alkaline phosphatase at 4 to 6 hours postoperatively was observed (354.97±95.00 IU/L) (Fig. 1). We used LHA to inhibit the second peak of alkaline phosphatase with LHA (Fig. 2), suggesting that this second peak is caused by the generation of Tissue Non-Specific Alkaline Phosphatase (TNSALP-type alkaline phosphatase) (17). Through isoenzyme analysis it was excluded that this postoperative rise of plasma alkaline phosphatase could be attributed to the rise of bIAP (Tab. II).

The mean value of serum bilirubin level in the placebo group increased from 10.87±6.66 μmol/L preoperatively to 13.28±6.13 μmol/L 24 hours postoperatively. In the bIAP group, this value increased significantly from 11.06±5.36 μmol/L preoperatively to 16.11±8.04 μmol/L 24 hours postoperatively.
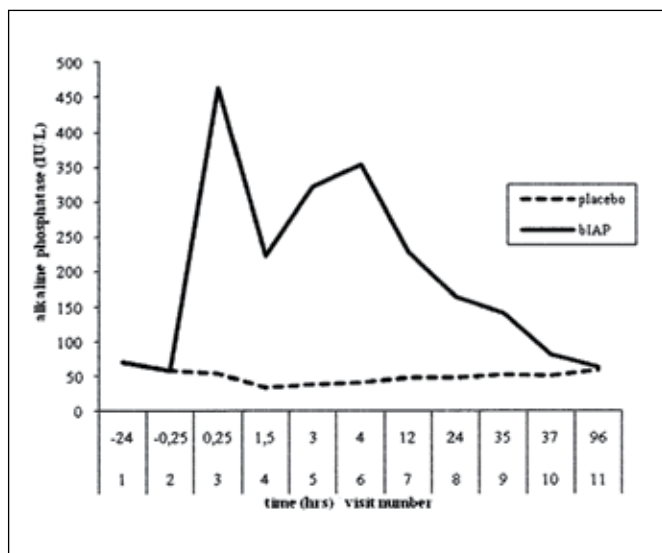
Fig. 1 - *Alkaline phosphatase levels at different time points perioperatively. Visit numbers correspond to the different time points.*
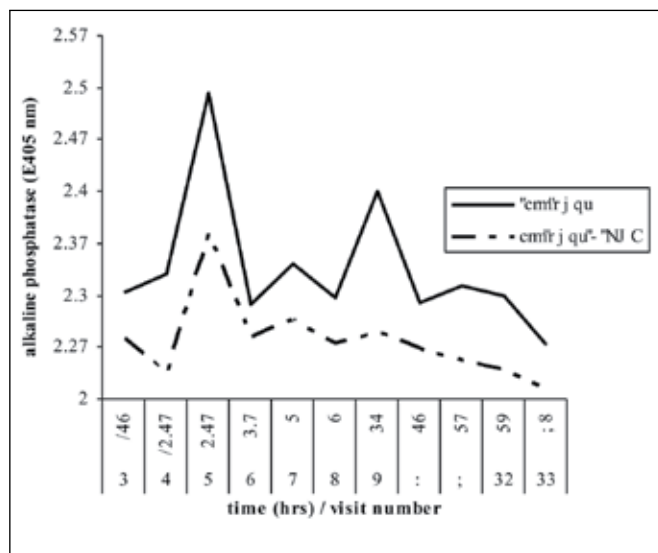

Fig. 2 - *Alkaline phosphatase levels at different time points perioperatively, either with or without LHA.*

DISCUSSION

This prospective study demonstrates that administration of bIAP in patients undergoing CABG with the use of CPB leads to the induction of endogenous alkaline phosphatase. This may lead to augmentation of the anti-inflammatory effect of bIAP in patients undergoing CABG with the use of CBP.

During CABG with the use of CPB, increased endotoxin translocation from the intestine occurs (18, 19). Moreover, it has been demonstrated that depending on CPB time and cross clamp time, ischemic insults occur followed by a local rise in nucleotides. Both ischemia-reperfusion mediated endotoxin and extra-cellular released nucleotides are potent pro-inflammatory triggers and are a substrate for both supplemental and endogenous alkaline phosphatase. Normally LPS travels with chyme and is taken up by both Kupffer cells and hepatocytes. This LPS is proposed to be predominantly detoxified through the activity of intestinal type alkaline phosphatase and systemically available alkaline phosphatase. In the absence of sufficient reactive alkaline phosphatase, its endotoxin clearance function may be suboptimal, resulting in further aggravation of endotoxin-mediated inflammatory effects. That is the reason why we supplemented bovine alkaline phosphatase in our study to combat endotoxin-induced inflammation in CABG with the use of CPB.

TABLE II - SERIAL SERUM VALUES OF ALKALINE PHOSPHATISE (AP)

Visit number	Total AP (IU/L)	Bone AP (IU/L)
	69.3±21.2	19.4±6.5
3	475±183.8	19±6.2
4	184±33.2	11±3.9
5	282±44.8	12±3.4
6	372±43.8	15.4±5.3
7	226±39.5	16.8±6.7
8	136±63.6	17.4±3.7

An interesting finding in this study was the difference in alkaline phosphatase kinetics in plasma between the bIAP and the placebo-treated group. In placebo-treated patients, a reduction of plasma alkaline phosphatase levels was measured 2 hours postoperatively. Normalized plasma levels were measured after 24 hours postoperatively. Reduction of plasma alkaline phosphatase levels after endotoxin administration levels was noted previously by Verweij et al in animal studies (20).

Kupffer cells may function to clear the alkaline phosphatase-LPS conjugates from the circulation, thereby reducing the total alkaline phosphatase levels (2, 20). This was also demonstrated in our study in the placebo group, who had an initial reduction of plasma alkaline phosphatase.

CABG with the use of CPB leads to a significant degree of hemodilution (21). In the APPIRED study (14), the mean hematocrit value dropped to a mean of 0.28. The reduction of alkaline phosphatase can be partially due to this hemodilution of approximately 40%. However, the reduction in alkaline phosphatase exceeds this 40% and thus a real clearance of alkaline phosphatase-LPS conjugates from the circulation must occur in our studied CABG population. This could be attributed in part to clearance of LPS conjugates from the circulation by Kupffer cells.

In the bIAP-treated group, an initial rise in alkaline phosphatase plasma level due to the bolus administration was observed. The kinetic profile of this plasma alkaline phosphatase level was compatible with the administered alkaline phosphatase with a physical half-life of about 10 minutes (9). Next to the initial increase in alkaline phosphatase level, a significant increase of plasma alkaline phosphatase at 4 to 6 hours postoperatively was observed. This second peak of alkaline phosphatase does not represent the intravenously administered bIAP, because this peak represents a much larger amount of systemic circulating alkaline phosphatase than the administered bIAP dose, which is calculated to give a doubling of normal plasma alkaline phosphatase levels during the perfusion time.

We sought to determine the origin of this endogenously released alkaline phosphatase in the bIAP-treated group and also to explain why only a bolus bIAP followed by continuous infusion leads to this endogenous alkaline phosphatase release.

This endogenous alkaline phosphatase is inhibited by L-homoarginine, which is known as an inhibitor of TNSALP. Thus likely indicates TNSALP with a physical half life of about 20 hours (17). As judged from the post-surgical amount of this TNSALP circulating, the most likely source is liver-type alkaline phosphatase, since it was demonstrated that the other abundant source (bone-type alkaline phosphatase) was not elevated. Taking the amount of hemodilution into account as well, it was interesting to find that even correcting for hemodilution, bilirubin level was significantly elevated in this group even after adjusting the results for hemodilution. This amplifies our conviction that this endogenous alkaline phosphatase is liver-type alkaline phosphatase.

This endogenous alkaline phosphatase release in patients undergoing cardiac surgery with the use of CPB, noted only in patients treated with a bIAP bolus followed by continuous infusion, is a unique finding that to our knowledge has not been described before. Pickkers et al (12) reported on the pharmacology of exogenously administered alkaline phosphatase in healthy volunteers and severe sepsis patients. They also used a dosing schedule with a bolus alkaline phosphatase followed by a continuous infusion in both the healthy volunteer group and the severe sepsis group. Although the total amount of administered alkaline phosphatase was higher in their patients, no endogenous alkaline phosphatase release was found in their group. Whether the use of CPB in our population is the cause of different results needs to be further investigated. Tuin et al demonstrated *in vitro* that LPS exposure to liver slices *in situ* results in increased mRNA for alkaline phosphatase expression with kinetics that are compatible with *de novo* synthesis (22).

Recently, a correlation between non-alcoholic fatty liver disease in obese persons and elevated liver enzymes has been demonstrated. Furthermore, a correlation between the amount of visceral abdominal tissue and elevated alkaline phosphatase levels has been demonstrated (23). In our study, although the mean BMI was significantly different between the two groups, no statistically significant difference between preoperative levels of alkaline phosphatase was found. This eliminates the correlation between the difference in the postoperative alkaline phosphatase release and the difference in the mean BMI between the two groups.

We hypothesize that alkaline phosphatase prophylaxis improves the defense mechanism against a new inflammatory insult by triggering the release of sustainable alkaline phosphatase in the circulation possibly because of *de novo* synthesis.

The surprising implication of this finding may have significant consequences. Alkaline phosphatase may act like an acute phase protein, where high levels of physiological active alkaline phosphatase have a protective anti-inflammatory effect. The preoperative plasma levels may predict clinical outcome in acute inflammation in a manner similar to that reported for high plasma anti-endotoxin antibody levels (24, 25). Patients might thus be protected by pretreatment with physiological active alkaline phosphatase which will elevate their endogenous physiological levels. However, as mentioned above, the mechanism of this postoperative rise of endogenous alkaline phosphatase observed in our study has yet to be elucidated and further studies are needed to investigate if this finding will have clinical advantage for CABG patients.

CONCLUSIONS

Intravenous bolus administration plus continuous infusion of alkaline phosphatase in patients undergoing coronary artery bypass grafting results in a subsequent rise in circulating plasma alkaline phosphatase levels 4 to 6 hours after start of surgery. The origin of this alkaline phosphatase is attributed to tissue non-specific alkaline phosphatase, most likely liver-type alkaline phosphatase. This endogenous alkaline phosphatase may play a role in the innate immune defense system.

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