Original article:

The Protective Effect of Azelnidipine for the Prevention of Heart Fibrosis Occurrence on Balb/c Mice with Iron Overload

Hadi Sarosa[1], Udin Bahrudin[2], Ag Soemantri[3], Siti Fatimah-Muis[4], Nur Arfian[5],Ichiro Hisatome[6]

Abstract:

Background: Iron overload can cause DNA oxidation which increase TGF β1, type 1 fibrilarprotein and myocardium fibrosis. Myocardium fibrosis is the main cause of death on the state of iron overload. The iron influx towards the cell during iron overload is still unknown, some research suggested LTCC acts as iron influx. This research aims to investigate the role of azelnidipine as type L calcium channel blocker, lowering TGF β1, collagen and myocardium fibrosis. ***Method:*** The research subjects consisted of 25 male Balb-C mice(8 weeks, 30-40mg) divided into 5 groups. Group 1 (NaCl+S) 0,3 cc Na Cl 0,9% (I.P) and drug solvent (Aquabidest, CMC and Nipagin) orally. Group 2Fe+S) 0.3 cc 1,5 mg Fe+sucrose (Venofer®) (I.P) and drug solvent (Aquabidest, CMC and Nipagin) orally. Group 3 (Fe+Dfx) 1,5 mg Fe+sucrose (Venofer®) (I.P) and deferasirox 20 mg/kg body weight/day orally, group 4 (Fe+Azl) 1,5 mg Fe+sucrose (Venofer®) (I.P) and azelnidipine 14 mg/day orally and group 5 (Fe+Dfx-Azl) 1,5 mg Fe+sucrose (Venofer®) (I.P) and mixture of deferasirox 20 mg/kg body weight/day and azelnidipine 14 mg/day orally. Fe-sucorse was diluted with NaCl 0.9 %. Intraperitoneal injection were administered intermittently for 60 days of treatment. ***Result:*** The highest Expression of TGF β, collagen I and fibrosis area fractions are in group Fe+S. The result of Post Hoc test between 2 treatment groups indicated that there were no difference in TGF β expression between groups NaCl+S with Fe+Dfx (P>0.05) , Fe+Dzl (P>0.05). There are no significant in collagen expression between groups NaCl+S with Fe+Dfx (P > 0.05) ,Fe+Dzl (P>0.05). ***Conclusion:*** Azelnidipine, LTCC have roles on the influx of iron into the myocardium, lowering TGF β, collagen Iexpressionsand myocardium fibrosis.

Keywords: Azelnidipine; iron overload; expression of collagen; myocardium fibrosis

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Introduction

Iron plasma overload is still a problem for people with thalassemia.Iron plasma overload occurred due to haemolysis, repeated transfusion and also due to the iron metabolism dysregulation. Administration of blood transfusion in β thalassemia will improve the accumulation of iron substance , which is 0.34 mg/kg body weight /day on patients with the need for 600 Red Packed Cell / 4 weeks [1].Iron plasma overload

1. Hadi Sarosa, Diponegoro University Postgraduate Program of Medical Sciences, Semarang, Indonesia & Departement of Physiology, Medical Faculty of Sultan Agung Islamic University Semarang, Indonesia
2. Udin Bahrudin, Department of Cardiology and Vascular Medicine, Diponegoro University Faculty of Medicine, Semarang, Indonesia
3. Ag Soemantri, Department of Pediatric, Diponegoro University Faculty of Medicine, Semarang, Indonesia
4. Siti Fatimah-Muis, Department of Nutrition, Diponegoro University Faculty of Medicine, Semarang, Indonesia
5. Nur Arfian, Departement of Anatomy, Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada Yogyakarta, Indonesia
6. Ichiro Hisatome, Division of Regenerative Medicine, Tottori University Graduate School of Medical Science, Yonago, Japan.

Correspondence to: Hadi Sarosa, Diponegoro University Postgraduate Program of Medical Sciences, Semarang, Indonesia & Departement of Physiology, Medical Faculty of Sultan Agung Islamic University Semarang, Indonesia. Postal address MedicalFaculty of UNIVERSITAS ISLAM SULTAN AGUNG ,Jalan Raya Kaligawe KM4, Semarang 50112, Central Java Indonesia. Email idahasoras@yahoo.com

cause the accumulation of iron in the internal organs. The accumulation of iron in cardiovascular muscle is cardiovascular complicationand the main cause of death on repeated transfusion[2]

On the physiological state, transferrin receptor 1 (TfR1) plays an important role in the process of iron influx to the intra cell. Transferrin receptor 1 (TfR1) which located in the cardiac cell wall is important in the influx of iron to the myocardium, however during the presence of high intra cell iron level, TfR1 expression will be suppressed[3–5]. On the state of high iron plasma, several studies suggested the type L calcium channel(L- type calsium channels/ LTCC)[2,6–11]while others stated that type T calcium channel *(T-type calsium channels*/TTCC)[12,13]are playing important roles in the influx of iron in the myocardium.

The accumulation of iron will cause cell damage due to the DNA oxidation. DNA oxidation triggers the apoptosis process and increasegrowth factor (TGF-β1, PDGF), cytokine and extracell matrices[14]. Cascadetransforming growth factor- β1 (TGF-β1) plays a major role in inducing fibroblast into myofibroblast and increasing MMP2 and MMP 9 expressions which cause fibrosis[14,15].Cardiomyopathy and heart failure/ cardiac arrest due to the myocardium fibrosis are the leading cause of death for iron plasma overload[2,9].

Management of iron accumulation to this day is not yet satisfactory, one of them is iron chelation. Deferasirox is the latest development of iron chelation, it has the ability to bind plasma iron and penetrate cell membranes to bind intra cell iron[16]. Deferasiroxis a tridentate molecule, forming 2 electrochemical bonds of Fe(2:1) ions which are quite stable, given orally once a day [17].The use of iron chelating drugs to eliminate iron accumulation in the heart is still not yet satisfactory

In a state of high plasma iron, several studies have shown L-type calcium channels (*L- type calsium channels*/LTCC) [11]while others suggested that T-type calcium channel *(T-type calsium channels*/TTCC) [13]plays an important role in the influx of iron into the myocardium. The type L calcium channel density is affected by estrogenic receptors, an increase in estrogen will reduce the number of type L calcium channels and reduce the entry of Ca^{2+} in the myocardium[18]. Azelnidipine is along acting third generation partition L type calcium channel, very soluble in fat[19]. Azelnidipine has the hydroxyl radical scavenger effect to reduce ROS, antioxidant and antifibrotic effects[20].

This research is going to look at the effect of azelnidipineon TGF β, collagen expression and iron overload model heart fibrosis on male experimental animals. Iron overload model is by administration of intraperitoneal iron sucrose in Balb/c mice intermittently.

Materials and Methods

Animal subjects

Experiments were conducted after approval by theethicalCommittee of the Faculty of Medicine, Diponegoro University, no 16/EC/H/FK-RSDK/V/2017. Male Balb/c mice (n = 25) age 8 weeks old, with 30-40 gr body weight were obtained from the Experimental Animal Care Unit (UPHP) LPPT of Gadjah Mada University. Mice were cagedmaintained by the Department of Biology, Faculty of Medicine Unissula, with a light-dark cycle of 12:12 hour, humidity : 50-60 %, temp : 24° -26°. Research subjects were randomly selected into 5 groups. Group 1 (NaCl+S) were given 0,3 cc Na Cl 0.9% intra peritonealand drug solvent (Aquabidest, CMC dan Nipagin) 0.5 cc by turns orally every day, group 2 (Fe+S)were given 1,5 mg (0.3 cc) Fe+ sucrose (Venofer®) and drug solvent 0.5 cc orally, group 3 (Fe+Dfx)were given 1,5 mg (0.3 cc) Fe+sucrose (Venofer®) and deferasirox 20 mg/kg body weight/ day orally, group 4 (Fe+Azl) were given 1,5 mg (0.3 cc) Fe+sucrose (Venofer®) andazelnidipine14 mg/day orally and group 5 (Fe+Dfx-Azl) were given 1,5 mg (0.3 cc) Fe+sucrose (Venofer®) and mixture of deferasirox 20 mg/kg body weight/ dayandazelnidipine 14 mg/dayorally, Fe-sucrose was diluted withNaCl 0.9 %, intraperitoneal injection and was done intermittent day. Mice got standard feeding and free access to water *ad libitum*. Mice were terminated at day 70 after operation

Heart harvesting

Before termination, the mice were anesthetized with pentobarbital (60 mg/kg ip), then the abdomen and thorax were opened. Heart were harvested and half ventricle was in RNA later ® for extraction RNA and other half was fixated in PFA 4% in PBS for 24 hour and paraffin was used embedded tissue process.

Histological analysis

Paraffin section with 4 mm thickness was analyzed. Paraffin section were deparaffinized and stained with Sirius Red to quantify the fibrosis interstitial fraction

area. Quantification of fibrosis area was done using Image J software, with 10 fields in each sample and with 400 x magnification.

Reverse Transcriptase PCR

RNA was extracted from heart tissue using FavorPrep™Tri-RNA Reagent (Favorgen, FATRR 001, Biotech Corp). cDNA was synthesized using ReverTra-Ace (TOYOBO Co, Ltd, TRT-101x10). Reverse transcriptase PCR (RT-PCR) was done for examining the expression of following gen : collagen 1 (forward, 5'-ATGCCGCGACCTCAAGATG-3'; reverse, 5'-GAGGCACAGACGGCTGAGTA-3'), TGF β1 (forward, 5'-TTCCGCTGCTACTGCAAGTCA-3'; reverse 5'-GGGTAGCGATCGAGTGTCCA-3'), Caspase 3 (forward 5'YCYGACTGGAAAGCCGAAACTC-3'; reverse, 5' TCCCACTGTCTGTCTCAATGCCAC-3'), GAPDH (forward, 5'-TTGCTGTTGAAGTCGCAGGAG-3'; reverse, 5'-TGTGTCCGTCGTGGATCTGA-3') was used reference.

Statistical Analysis

Data were presented as mean ± SD collagen and TGF β level and fraction area fibrosis were analysed with ANOVA test and continued with Post Hoc test

Ethical clearance: This research study was approved by ethics committee of Medical Faculty of Sultan Agung Islamic University Semarang, Indonesia.

Results

On group Fe+S the highest average of TGF β 1 and collagen I expression. The lowest average of TGF β expression was on group Fe+Dfx and collagen expression the lowest expression was on group Fe+Azl

Table1 : TGF β1expression on various treatment groups on Balb-c mice

Mice	NaCl + S	Fe + S	Fe + Dfx	Fe + Azl	Fe + Dfx-Azl
1	1.56	1.52	1.30	1.37	1.36
2	1.37	1.46	1.43	1.43	1.40
3	1.39	1.45	1.26	1.24	1.43
4	1.50	1.86	1.25	1.48	1.58
5	1.52	1.75	1.36	1.45	1.67
Mean	1.47±0.08	1.61±1.86	1.32±0.06	1.40±0.1	1.49±0.13

Table 2 : Collagen I expression on various treatment groups on Balb-c mice

Mice	NaCl + S	Fe + S	Fe + Dfx	Fe + Azl	Fe + Dfx-Azl
1	1.20	1.32	1.13	1.25	1.22
2	1.21	1.24	1.12	1.11	1.19
3	1.16	1.21	1.07	0.96	1.16
4	1.23	1.56	1.04	1.16	1.35
5	1.30	1.50	1.17	1.20	1.39
Mean	1.22±0.05	1.37±0.16	1.11±0.05	1.14±0.11	1.26±0.10

The administration of Fe indicated that there was increasing expression of TGF β 1and collagen I expression which will cause damage on heart muscle and cause fibrosis. The results of ANOVA test, there is significant difference on TGF β 1expression ($p < 0.05$) and collagen I expression ($p < 0.01$). The result of Post Hoc test between 2 treatment groups indicated that there were no difference in TGF β expression between groups NaCl+S with Fe+Dfx (P>0.05), Fe+Dzl (P>0.05). There are no significant in collagen expression between groups NaCl+SwithFe+Dfx ($P > 0.05$) ,Fe+Dzl (P>0.05).

Discussion

Myocardium is composed from several types of cells, cardiomyocyte, cardiofibroblast and endothelial cell. Cardiofibroblast generate cytokines, and growth factors and have the function to preserve the structural and functional integrities of the extracellular matrices. The extracellular matrix has a very important role as the mechanical and chemical properties between cardiomyocytes, cardiofibroblast cells and blood vessels [14]. The myocardial extracellular matrix is woven between cells arranged by proteins, fibrous hidden in materials similar to gels composed of complex carbohydrates. A dilute gel is called interstitial fluid, which is the space between blood vessels and tissue cells which are part of nutrients, residual results, and materials that dissolve in the air [21]. The extracellular matrix composes three components of macromolecules namely glycosaminoglycan, fibrous protein collagen and glycoprotein [22].Inflamation[23], oxidative stress[24] and hypoosmolar hyperhydration[25]arecontributors to cardiomyocyte impairment. Damage to the myocardium increases several growth factors (TGF β1, PDGF), cytokines (TNF α, IL1β, IL6), extracellular matrix and causes cardiofibroblast differentiation to myocardiofibrobast. Myocardiofibroblasts have contraction ability, can move and produce more extracellular matrix proteins [14]. Cardiofibroblast hyperactivity and myocardiofibroblasts increase the production of collagen I, II fibilar proteins and

extracellular matrix deposits[15]. Cardiofibroblast accumulation and accumulation of collagen fibrillary proteins that are maternity, supported by tissue and myocardial function[14,26,27].

This study indicated that that intraperitoneal administration of Fe 1,5 mg intraperitoneal by intermittent days can increase the TGF β1,Collagen1 expression and myocardium fibrosis.

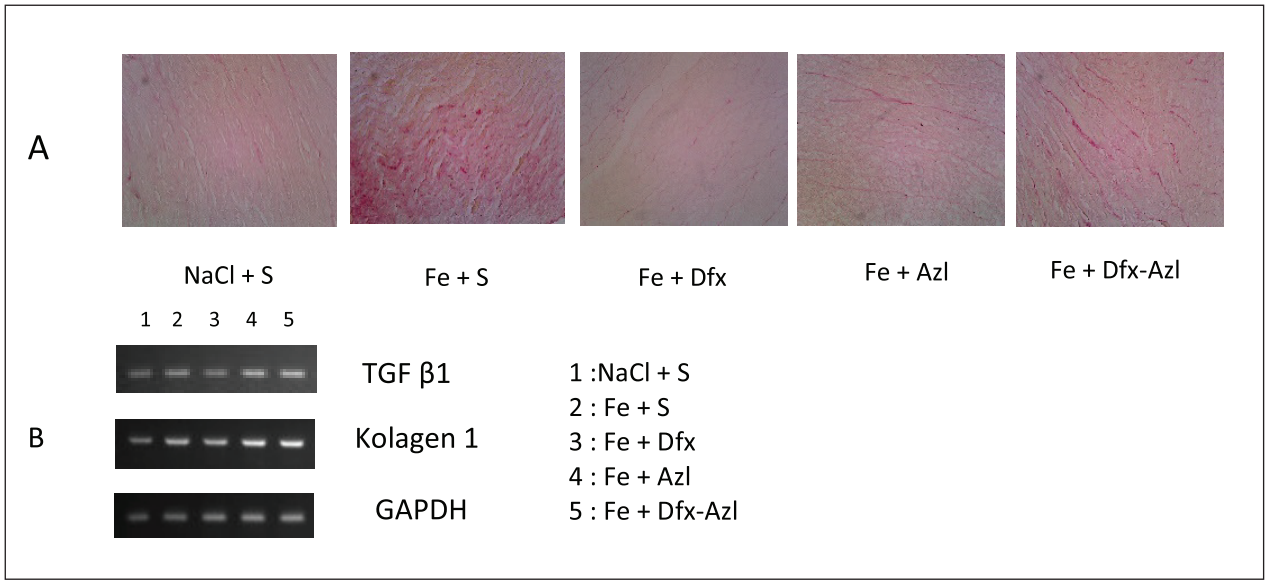

Figure 1 : (A)Histochemistry examination with Sirius Red (SR) staining indicated that group Fe+S with the most fibrosis area fractions, no significant difference of area fractions (%) NaCl + S with Fe + Azl

(B) reverse transcriptase PCR (RT-PCR) TGF β1, Collagen I and GAPDH, and densitometry analysis with Image J software indicated that there is no difference (P>0.05) Group Fe+Azl vs NaCl+S, Fe+Azl vs Fe+Dfxdan Fe+Azl vs Fe+Dfx-Azl. There is significant difference (P<0.05) GroupFe+Azl Vs Fe+S.

Myocardial iron deposits result in oxidative stress due to an increase in the hydroxyl radical prooxide. Increased hydroxyl radicals increase peroxidase and damage to lipids, proteins and DNA[28]. DNA damage is measured by expression of growth factor (TGF-β1). Cascade transforming growth factor- β1 (TGF- β1) induces fibroblasts into myofibroblasts and increases the expression of collagen fibilar proteins, which results in fibrosis [14,15].

L- type calsium channels (LTCC) are mostly in the myocardium, play an important role during calcium entry as triggers of heart muscle contraction, regulation of the duration of action potential and regulation of gene expression[29]. The relationship between chronic heart failure and serum calcium[30]. In the state of iron overload, LTCC plays a role in the influx of iron into the heart muscle[2,6–11]. The lowest of collagen expression average were in the Fe-Azl group, Azelnidipine is type L Ca Channel blocker which is an antihypertensive drug with better effect of anti-inflammatory and anti-oxidant than amlodipine, furthermore, it has the antifibotic effect by inhibiting TGF-β1 in the liver[20], and inhibits apoptosis by reducing cytochrome C levels in HL-1 cardiomyocytes[31]

Azelnidipine is an anti-hypertension that can prevent heart damage. Azelnidipine has better anti inflammatory and antioxidant effects than amlodipine [32]antifibrotic effects by inhibiting TGF-β1 in the liver [20], and inhibiting apoptosis by reducing cytochrome C levels in HL-1 cardiomyocytes[31]. Cardiofibroblast hyperactivity and myocardial fibroblasts increase the production of collagen I, II fibilar proteins and extracellular matrix deposits [15]. Fibrillar collagen type I and II are the most common components of the myocardial extracellular matrices[14]. TGF β1 pathway is an important pathway for collagen production [33]and where fibrosis takes place [15].

L-type calcium channels (LTCC) play a role in the entry of Fe2+ [2,6–11].This study shows that administration of azelnidipine has a protective effect on the occurrence of myocardial fibrosis, lowering TGF β1 and collagen Iexpression . L Type Calciumchannel (LTCC) plays a role in the entry of iron into the cell.

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Conflict of Interest:

The authors declared that they have no conflict of interest.

Contribution of Authors:

Data gathering and idea owner of this study: HadiSarosa

Study design: HadiSarosa

Data gathering:HadiSarosa, Udin Bahrudin, Nur Arfian

Writing and submission of manuscript: Hadi Sarosa, Ag Soemantri, Siti Fatimah-Muis, Udin Bahrudin, Nur Arfian, Putri R Ayuningtyas

Editing and approval of final draft: HadiSarosa, Ag Soemantri, Siti Fatimah Muis, Udin Bahrudin, Ichiro Hisatome

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