Original article:

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

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Abstract:

Background: Iron overload can cause DNA oxidation which increase TGF β1, type 1 fibrilar protein 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, 30-40 mg) divided into 5 groups. Group 1 (NaCl + S) 0.3 cc NaCl 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-sucrose 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 expressions and myocardium fibrosis.

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

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. Iron plasma overload

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cause the accumulation of iron in the internal organs. The accumulation of iron in cardiovascular muscle is cardiovascular complication and the main cause of death on repeated transfusion.

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. On the state of high iron plasma, several studies suggested the type L calcium channel (L-type calcium channels/LTCC) while others stated that type T calcium channel (T-type calcium channels/TTCC) 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 increase growth factor (TGF-β1, PDGF), cytokine and extracellular matrices. Cascade transforming growth factor-β1 (TGF-β1) plays a major role in inducing fibroblast into myofibroblast and increasing MMP2 and MMP9 expressions which cause fibrosis. Cardiomyopathy and heart failure/cardiatic arrest due to the myocardium fibrosis are the leading cause of death for iron plasma overload.

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. Deferasirox is a tridentate molecule, forming 2 electrochemical bonds of Fe(2⁺) ions which are quite stable, given orally once a day. 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 calcium channels/LTCC) while others suggested that T-type calcium channel (T-type calcium channels/TTCC) 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 Ca²⁺ in the myocardium. Azelnidipine is along acting third generation partition L type calcium channel, very soluble in fat. Azelnidipine has the hydroxyl radical scavenger effect to reduce ROS, antioxidant and antifibrotic effects.

This research is going to look at the effect of azelnidipine on 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 the ethical Committee of the Faculty of Medicine, Diponegoro University, no 16/EC/H/FK-RSDK/IV/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 caged maintained by the Department of Biology, Faculty of Medicine Unissula, with a light-dark cycle of 12:12 hour, humidity : 50-60 %, temp : 24°C -26°C. Research subjects were randomly selected into 5 groups. Group 1 (NaCl+S) were given 0,3 cc NaCl 0,9% intra peritoneal 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®) and azelnidipine14 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/day and azelnidipine 14 mg/day orally. 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.
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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'-GAGGCACACCGGCTGAGTA-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

Table 1 : TGF β1expression on various treatment groups on Balb-c mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>NaCl + S</th>
<th>Fe + S</th>
<th>Fe + Dfx</th>
<th>Fe + Azl</th>
<th>Fe + Dfx-Azl</th>
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<tbody>
<tr>
<td>1</td>
<td>1.56</td>
<td>1.52</td>
<td>1.30</td>
<td>1.37</td>
<td>1.36</td>
</tr>
<tr>
<td>2</td>
<td>1.37</td>
<td>1.46</td>
<td>1.43</td>
<td>1.43</td>
<td>1.40</td>
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<td>1.24</td>
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</tr>
<tr>
<td>4</td>
<td>1.50</td>
<td>1.86</td>
<td>1.25</td>
<td>1.48</td>
<td>1.58</td>
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<tr>
<td>5</td>
<td>1.52</td>
<td>1.75</td>
<td>1.36</td>
<td>1.45</td>
<td>1.67</td>
</tr>
<tr>
<td>Mean</td>
<td>1.47±0.08</td>
<td>1.61±1.86</td>
<td>1.32±0.06</td>
<td>1.40±0.1</td>
<td>1.49±0.13</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Mice</th>
<th>NaCl + S</th>
<th>Fe + S</th>
<th>Fe + Dfx</th>
<th>Fe + Azl</th>
<th>Fe + Dfx-Azl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.20</td>
<td>1.32</td>
<td>1.13</td>
<td>1.25</td>
<td>1.22</td>
</tr>
<tr>
<td>2</td>
<td>1.21</td>
<td>1.24</td>
<td>1.12</td>
<td>1.11</td>
<td>1.19</td>
</tr>
<tr>
<td>3</td>
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<td>1.21</td>
<td>1.07</td>
<td>0.96</td>
<td>1.16</td>
</tr>
<tr>
<td>4</td>
<td>1.23</td>
<td>1.56</td>
<td>1.04</td>
<td>1.16</td>
<td>1.35</td>
</tr>
<tr>
<td>5</td>
<td>1.30</td>
<td>1.50</td>
<td>1.17</td>
<td>1.20</td>
<td>1.39</td>
</tr>
<tr>
<td>Mean</td>
<td>1.22±0.05</td>
<td>1.37±0.16</td>
<td>1.11±0.05</td>
<td>1.14±0.1</td>
<td>1.26±0.10</td>
</tr>
</tbody>
</table>

The administration of Fe indicated that there was increasing expression of TGF β 1 and 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+S with Fe+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. Inflammation23, oxidative stress24 and hypoosmolar hyperhydration25 are contributors to cardiomyocyte impairment. Damage to the myocardium increases several growth factors (TGF β1, PDGF), cytokines(TNFα, 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 fibrilar proteins and
extracellular matrix deposits\textsuperscript{15}. Cardiofibroblast accumulation and accumulation of collagen fibrillary proteins that are maternity, supported by tissue and myocardial function\textsuperscript{14,26,27}.

This study indicated that that intraperitoneal administration of Fe 1.5 mg intraperitoneal by intermittent days can increase the TGF \(\beta_1\), Collagen I expression and myocardium fibrosis.

<table>
<thead>
<tr>
<th>A</th>
<th>NaCl + S</th>
<th>Fe + S</th>
<th>Fe + Dfx</th>
<th>Fe + Azl</th>
<th>Fe + Dfx-Azl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TGF (\beta_1)</td>
<td>1 : NaCl + S</td>
<td>2 : Fe + S</td>
<td>3 : Fe + Dfx</td>
<td>4 : Fe + Azl</td>
</tr>
<tr>
<td>2</td>
<td>Kolagen 1</td>
<td>5 : Fe + Dfx-Azl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>GAPDH</td>
<td></td>
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</table>

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 \(\beta_1\), 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\textsuperscript{28}. DNA damage is measured by expression of growth factor (TGF-\(\beta_1\)). Cascade transforming growth factor- \(\beta_1\) (TGF- \(\beta_1\)) induces fibroblasts into myofibroblasts and increases the expression of collagen fibrillar proteins, which results in fibrosis \textsuperscript{14,15}.

L-type calcium 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\textsuperscript{29}. The relationship between chronic heart failure and serum calcium\textsuperscript{30}. In the state of iron overload, LTCC plays a role in the influx of iron into the heart muscle\textsuperscript{3,6–11}. 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 amloidipine, furthermore, it has the antibibotic effect by inhibiting TGF-\(\beta_1\) in the liver\textsuperscript{20}, and inhibits apoptosis by reducing cytochrome C levels in HL-1 cardiomyocytes\textsuperscript{31}. Azelnidipine is an anti-hypertension that can prevent heart damage. Azelnidipine has better anti-inflammatory and antioxidant effects than amloidipine\textsuperscript{32} antifibrotic effects by inhibiting TGF-\(\beta_1\) in the liver\textsuperscript{20}, and inhibiting apoptosis by reducing cytochrome C levels in HL-1 cardiomyocytes\textsuperscript{31}. Cardiofibroblast hyperactivity and myocardial fibroblasts increase the production of collagen I, II fibrilar proteins and extracellular matrix deposits\textsuperscript{15}. Fibrillar collagen type I and II are the most common components of the myocardial extracellular matrices\textsuperscript{14}. TGF \(\beta_1\) pathway is an important pathway for collagen production\textsuperscript{32} and where fibrosis takes place\textsuperscript{15}.

L-type calcium channels (LTCC) play a role in the entry of Fe2+\textsuperscript{2,6–11}. This study shows that administration of azelnidipine has a protective effect on the occurrence of myocardial fibrosis, lowering TGF \(\beta_1\) and collagen I expression. L-type Calciumchannel (LTCC) plays a role in the entry of iron into the cell.
Acknowledgement

Source of Funding

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We would like to appreciate to those who are participating in this study. This work was performed in partial fulfillment of the requirements for Doctoral of Faculty of Medicine Diponegoro University.

Conflict of Interest:

The authors declared that they have no conflict of interest.

Reference:

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