Physiological Studies on the Risk Factors Responsible for Atherosclerosis in Rats

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Abstract: Despite the well established correlation between hypercholesterolemia and coronary artery disease (CAD), a substantial body of evidence challenge this relationship. The study aimed to examine whether hyperlipidemia per se constitutes the principal risk factor for atherosclerosis or just a coordinator to other critical mediators. Hyperlipidemia was produced by feeding rats with high-fat diet for two months. The occurrence of hyperlipidemia was determined by measuring lipid profile. The hyperlipidemic rats were subdivided into two groups i) hyperlipidemic rats ii) hyperlipidemic rats injected with single dose of *Escherichia coli* (*E. Coli*) (and kept for two weeks to develop bacteremia and its subsequent effects. Result showed that hyperlipidemia significantly increased total cholesterol, triglycerides, low density lipoprotein (LDL) and homocysteine levels, whereas decreased high density lipoprotein cholesterol (HDL) levels. Moreover, hyperlipidemia induced mild oxidative stress in terms of elevated levels of malondialdehyde (MDA) and nitric oxide (NO) and decreased level of reduced glutathione (GSH) in blood. In addition, hyperlipidemic rats exhibited high plasma viscosity, altered hematological indices and caused histological abnormalities manifested as perivascular hemorrhage, vacuolation of the tunica media and minor thickening in aorta wall. Bacteremia provoked inflammatory reactions and oxidative stress, elevated plasma homocysteine and caused noticeable considerable thickening of media-intema layer suggesting the commencement of atherosclerosis. Hyperlipidemic-bacteremic rats showed an additive effect. The study indicated that although hyperlipidemia is an apparent risk factor, homocysteinemia, the inflammatory component and the oxidative stress emerge to be the underlying mechanisms of atherosclerosis pathogenesis. [Nature and Science 2010;8(5):144-151]. (ISSN: 1545-0740).

Key words: Hyperlipidemia- Bacteremia- inflammation- Atherosclerosis

1- Introduction
Clinical studies indicated that hypercholesterolemia is an essential risk factor for coronary artery disease (CAD), where low-density lipoprotein (LDL) cholesterol plays a major role in the atherosclerosis and pathogenesis of CAD and other vascular diseases (Trubelja et al, 2005). Furthermore, several studies showed that hyperlipidemia induces oxidative stress and the oxidative modification of lipoproteins in vessel walls might play a key role in atherogenesis (Wittenstein et al., 2002). Noteworthy, there is a substantial body of evidence challenging the theoretical relationship between dietary cholesterol and CAD. In addition, the relationship between cholesterol in foods and cholesterol in the blood has never been conclusively established and remains a topic of considerable debate. Moreover, hypercholesterolemia has been shown to have a protective effect against atherosclerosis (Ravnkov 2003).

On the other side, elevated C-reactive protein level, an important marker of inflammation, has been acknowledged as an independent risk factor for the development of atherosclerosis and ischemia even in normal cholesterol levels (Collins et al., 2004). In addition, the observation that lipid lowering agents other than statins, such as fibrates, resins, or diet has no impact on stroke incidence (Collins et al., 2004), might indicate that in absence of the inflammatory reactions and oxidative stress, hyperlipidemia is not the prominent risk factor for stroke (Ridker et al., 2000). However, it seems that the interaction between the hemorheological variables (plasma and whole blood viscosities, hematocrit, red blood cell aggregation) and hematological parameters [plasma fibrinogen and von Willebrand factor (vWf)], and platelet aggregation are acknowledged to play roles in atherosclerotic heart diseases (Kesmarky et al., 2006).

Escherichia coli (*E. coli*), a Gram negative bacteria, is a common cause of infections in all populations and countries of the world (Al-Hasan et al., 2009). *E. coli* escape the intestinal tract and enter the abdomen through an ulcer, a ruptured appendix, or a surgical error. This leads to peritonitis and elicits a vigorous immune system response and consequently cause bacteremia, sepsis (blood poisoning) and septic shock, which has a relatively high mortality rate (Ruthrauff et
al., 2009). Endotoxin or LPS, a component of the wall of Gram-negative bacteria with significant proinflammatory properties, is a primary initiator of inflammatory and hemodynamic consequences of sepsis [Wang et al., 2007].

The aim of the present study was to evaluate the hematological, biochemical, histopathological effect of hyperlipidemia and inflammation alone or in combination in rat. This was achieved by measuring the lipid profile, the inflammatory parameters (C-reactive protein, erythrocyte sedimentation rate, (ESR); and the differential count of the white blood cells and platelets) and the plasma viscosity. In addition, the oxidative stress parameters (nitric oxide, NO; reduced glutathione (GSH); malondialdehyde (MDA) and homocysteine in plasma of different groups were determined. Moreover, the histopathological studying and measuring the thickness of aorta in different treatment were carried out.

2- Materials and Methods

Male adult Sprague Dawley rats (150-200 g) were kindly provided from our breeding center of National Organization for Drug Control and Research (NODCAR) and kept for a week for acclimatization under normal conditions and constant temperature (25±1°C) with ad libitum of water and food. All experiments were carried out in accordance with research protocols established by the animal care committee of the National Organization for Drug Control and Research, Egypt.

All chemicals used were of analytical grade. Solution of pathogenic strain of Escherichia coli (E. coli) bacteria was kindly provided by Mrs. Eqbal Abdel Hafez, microbiology department, NODCAR. The solution was diluted with saline to colony forming units 2x10^7 CFU/mL. Each rat intraperitoneally administered 0.5 ml containing 10^7 CFU.

2.1. Induction of hyperlipidemia:

Hyperlipidemia in rats was done according to the method of Gröne et al. [1989]. In briefly, hyperlipidemia was induced by feeding the animals high-fat diet [40%] fat / cholesterol (5%)] for two months. The high-fat diet contained cholic acid (0.35%) to enhance the enteral absorption of lipids. The occurrence of hyperlipidemia was determined by measuring lipid profile(total cholesterol, triglycerides and HDL). The hyperlipidemic animals were only used.

2.2. Experimental Design

A number of thirty hyperlipidemic rats were divided into two groups as follows.

- Positive control, (n=10)
- Hyperlipidemic- bacteremic group: twenty hyperlipidemic rats injected with single dose of (10^7 CFU/rat) E.Coli and kept for two weeks. The group is comprised of twenty rat, because from previous studied the mortality rate of bacteremia amounted to 50%. In addition, a number of 28 normal diet fed animals were divided into two groups as follows:
  - Bacteremic group (normal diet-fed animals were injected with single dose of E.Coli (10^7 CFU/rat) and kept for two weeks (n=20). The group is comprised of twenty rat, because from previous studied the mortality rate of bacteremia amounted to 50%.
  - A group of animals fed normal diet served as normal control group (n=8).

The animals were sacrificed by decapitation; the blood samples were collected into heparinized tubes and centrifuged at 3000 r.p.m for 10 min. for plasma separation.

2.3. Methods

Determination of total cholesterol, triglycerides, and high density lipoprotein (HDL) were analyzed using commercial available kits (STANBIO Lab. TX, USA). Low density lipoprotein was calculated mathematically by Friedwald’s formula (1972). Determination of reduced glutathione, homocysteine, malondialdehyde and nitric oxide (as total nitrite and nitrate) levels were determined by HPLC methods according to the Jayatilleke and Shaw (1993), Or-Rashid et al. (2000), Karatepe (2004) and Everett et al. (1995) respectively. Erythrocyte sedimentation rate, leucocytes differential and platelets count were carried out using the method adapted by Simmons and Bernard (1997). Plasma viscosity was determined using BROOK FIELD DV-III ULTRA Programmable Rheometer-USA. CRP was detected with ELISA kit for rat (Genway Biotech, Inc., CA, USA), with the normal level being less than 0.5 mg/ml.

2.4. Histopathological examination and Morphometric Measurements:

Histopathology was carried out according to Carleton and Drury (1973). Cuts were made at a right angle to aorta and fixed in 10% buffered formalin. Sections of 4 μm thickness were stained with hematoxylin and eosin, aorta with uniform throughout its circumference have been selected for morphometric measurements. Morphometric measurements of thickness of cross-sectionally cut aorta were obtained under x 450 magnification with a calibrated filar micrometer.

2.5. Statistical analysis
Data presented as means ± SE. One-way ANOVA followed by LSD test were used to evaluate significant differences from the control and hyperlipidemic groups. Statistical processor system support (SPSS) for Windows software, release 10.0 (SPSS, Inc, Chicago, IL) was used.

3. Results
3.1. Biochemical investigation.

Feeding of animals with high fat-diet for two month significantly increased the levels of blood total cholesterol, triglycerides and LDL and decreased the level of HDL in both the hyperlipidemic group and hyperlipidemic-bacteremic group in comparison to control group. Bacteremic group didn’t differ from control group (Table 1). Hyperlipidemic, bacteremic, and hyperlipidemic-bacteremic groups exhibited low count of blood platelets, and total and differential (lymphocyte and monocyte) white blood corpuscles (WBC) in comparison to control (Table 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Hyperlipidemic</th>
<th>Hyperlipidemic-Bacteremic</th>
<th>Bacteremic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (Mg/dl)</td>
<td>71.000 ± 1.852</td>
<td>105.750 ± 3.081*</td>
<td>93.000 ± 2.847*</td>
<td>71.375 ± 1.679*</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>62.375 ± 2.828</td>
<td>82.375 ± 2.095*</td>
<td>73.750 ± 4.092*</td>
<td>65.625 ± 1.133*</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>42.875 ± 2.158</td>
<td>35.625 ± 1.752*</td>
<td>36.500 ± 1.439</td>
<td>40.625 ± 1.475</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>15.650 ± 1.487</td>
<td>53.650 ± 3.659*</td>
<td>41.750 ± 3.760*</td>
<td>17.625 ± 2.884*</td>
</tr>
</tbody>
</table>

* Significant different from control group at P< 0.05.
+ Significant different from hyperlipidemic group at P< 0.05

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Hyperlipidemic</th>
<th>Hyperlipidemic-Bacteremic</th>
<th>Bacteremic</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs (x10^3/mm^3)</td>
<td>11.179 ± 0.328</td>
<td>9.778 ± 0.289*</td>
<td>6.330 ± 0.219*</td>
<td>4.534 ± 0.245 **</td>
</tr>
<tr>
<td>Lymphocyte (x10^3/mm^3)</td>
<td>6.077 ± 0.297</td>
<td>4.245 ± 0.244*</td>
<td>2.116 ± 0.165*</td>
<td>1.069 ± 0.712 **</td>
</tr>
<tr>
<td>Monocyte (x10^3/mm^3)</td>
<td>0.742 ± 0.034</td>
<td>0.722 ± 0.024</td>
<td>0.438 ± 0.018*</td>
<td>0.257 ± 0.121 **</td>
</tr>
<tr>
<td>Blood Platelets (x10^3/mm^3)</td>
<td>0.381 ± 0.01</td>
<td>0.350 ± 0.009*</td>
<td>0.299 ± 0.006*</td>
<td>0.243 ± 0.005 **</td>
</tr>
</tbody>
</table>

* Significant different from control group at P< 0.05.
+ Significant different from hyperlipidemic group at P< 0.05

Hyperlipidemic, bacteremic and hyperlipidemic-bacteremic groups exhibited heightened plasma viscosity in comparison to control. In addition, the ESR was higher in hyperlipidemic, hyperlipidemic-bacteremic and bacteremic groups in an ascending order respectively in comparison to control. On the other hand, bacteremic and hyperlipidemic-bacteremic groups gave strong positive indication for C-reactive protein, while hyperlipidemic rats gave weak positive indication (Table 3). Data in Table 4 showed that the hyperlipidemic, bacteremic and hyperlipidemic-bacteremic groups showed elevated level of homocysteine, NO and MDA and decreased GSH level in comparison to control. The hyperlipidemic-bacteremic group showed an additive effect.
3.1. Histopathological and morphometric investigations.

Figure 1 and Table 5 showed normal architecture of aorta in normal control animals and normal aorta thickness. Aorta of hyperlipidemic animals showed hemorrhage in perivascular tissue (Fig. 2), with vacuolation in the cells of the tunica media (Fig. 3), and minor increase in the thickness of aorta wall (Table 5). Bacteremic group showed desquamation in the lining endothelium while the tunica media was hyalinized (Fig. 4) and exhibited remarkable increase in the thickness of aorta wall (Table 5). Histopathological examination of hyperlipidemic-bacteremic animals showed hemorrhage in the perivascular tissue (Fig. 5), and oedema in the tunica adventitia (Fig. 6), accompanied with thickening of aorta (Table 5).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Effect of high fat- diet and Escherichia coli (10^7 CFU/ rat i.p) treatment alone and in combination on plasma viscosity, erythrocyte sedimentation rate, and C-reactive protein in rat.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>Viscosity (CPS)</td>
<td>1.188 ± 0.295</td>
</tr>
<tr>
<td>Erythrocyte sedimentation Rate (ESR)-1 hr (mm/hr)</td>
<td>6.625 ± 0.420</td>
</tr>
<tr>
<td>Erythrocyte sedimentation Rate (ESR)-2 hr (mm/hr)</td>
<td>12.250 ± 0.526</td>
</tr>
<tr>
<td>C-reactive protein (mg/ml)</td>
<td>0.475 ± 0.033</td>
</tr>
</tbody>
</table>
* Significant different from control group at P< 0.05.
+ Significant different from hyperlipidemic group at P< 0.05.

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Effect of high fat- diet and Escherichia coli (10^7 CFU/ rat i.p) treatment alone and in combination on plasma reduced glutathione, nitric oxide, homocysteine and malondialdehyde in rat.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>Reduced glutathione (mg/ml)</td>
<td>41.583 ± 2.160</td>
</tr>
<tr>
<td>Nitric oxide (nmol/ml)</td>
<td>24.750 ± 0.491</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>6.831 ± 0.236</td>
</tr>
<tr>
<td>Malondialdehyde (nmol/L)</td>
<td>61.344 ± 3.417</td>
</tr>
</tbody>
</table>
* Significant different from control group at P< 0.05.
+ Significant different from hyperlipidemic group at P< 0.05.

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Effect of high fat- diet and Escherichia coli (10^7 CFU/ rat i.p) treatment alone and in combination on aorta wall thickness in rat.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>Aorta Wall Thickness (mm)</td>
<td>0.071 ± 0.002</td>
</tr>
<tr>
<td>% of control</td>
<td>100.00%</td>
</tr>
</tbody>
</table>
* Significant different from control group at P< 0.05. n=6
+ Significant different from hyperlipidemic group at P< 0.05.
Fig. (1): Transverse section of aorta of control rat showing normal histological structure of the tunica intima (I), media (M) and adventitia (A). (H & E X 160)

Fig. (2): Transverse section of aorta of hyperlipidemic rat showing perivascular hemorrhage (H). (H&E X 40).

Fig. (3): Transverse section of aorta of hyperlipidemic rat showing vaculation in the cells of tunica media (arrow). (H & E X 160).

Fig. (4): Transverse section of aorta of Escherichia coli (10⁷ CFU/ rat, i.p) treated rat showing focal desquamation of the endothelial cells lining the intima (arrow) with hyalinization in the tunica media (M). (H & E X 160).

Fig. (5): Transverse section of aorta of hyperlipidemic and Escherichia coli (10⁷ CFU/ rat, i.p) treated rat showing perivascular hemorrhage (H). (H & E X 160).

Fig. (6): Transverse section of aorta of hyperlipidemic and Escherichia coli (10⁷ CFU/ rat, i.p) treated rat showing oedema in the adventitia (A) (H & E X 64).
4. Discussion

4.1. Biochemical, hematological and histological effects of hyperlipidemia

The present data showed that feeding rats with high-fat diet for two months induced hyperlipidemia. Hyperlipidemic animals exhibited high plasma viscosity, elevated plasma homocysteine level, mild oxidative stress, altered hematological indices and thickening in media-intima layer. In agreement to the present results, a recent study showed that feeding of albino rats with high fat diet increased atherogenic indices and induced vascular endothelial dysfunction in isolated aorta of atherogenic-diet rats (Nakagawa et al., 2009). Furthermore, feeding of rats with high fat diet and a single dose of vitamin D (3) produced atherosclerosis in Sprague-Dawley rats, and induced hemorrhological and histopathological abnormalities in the atherogenic diet fed rat model. (Wu et al., 2009). Moreover, Chen et al. (2009) studied the mechanical properties of aortic artery in rats with atherosclerosis (AS), where the relationship between mechanical measurements and collagen concentration was evaluated. A close relationship between the mechanical constants and the percentage of elastin and collagen content was observed. It was concluded that mechanical remodeling in aortic artery of AS might be related with histological remodeling.

Besides, it is likely that the elevated plasma viscosity might constitute a risk factor in hyperlipidemic subjects (Cecchi et al., 2006). In accordance to present finding, previous studies indicated that hyperlipidemia increased the levels of lipid parameters and induces oxidative stress and initiated atherosclerosis (Wittenstein et al., 2002, Collins et al., 2004). On the other hand, severe hyperlipidemia in patients with glycogen storage disease type Ia (GSD Ia) failed to provoke premature atherosclerosis (Wittenstein et al., 2002), which might indicate that in absence of the inflammatory reactions and oxidative stress, hyperlipidemia was not the prominent risk factor for stroke. In accordance to the present findings, hyperhomocysteinemia (hhCy) per se has been recognized as a new risk factor for cardiovascular diseases, independent of plasma lipid levels or other factors (Li et al., 2002). Moreover, hyperhomocysteinemia has been found to increase carotid intima-media thickness which is a marker of early atherosclerosis (Spence 2002).

The present histopathological findings indicated that hyperlipidemia causes mild structural abnormalities manifested as thickened media-intima layer in comparison to control group; this effect might be due to accumulation of fatty vacuoles cells in the tunica media which resulted into narrowing of the aorta diameter. In accordance, a previous study indicated that hyperlipidemia causes accumulation of fatty plaque deposits in the arteries and aggravate narrowing of the arterial diameter, which restricts blood flow to vital organs (Rioufol & Finet 2002).

4.2. Biochemical, hematological and histological effects of bacteremia

Bacteremic group exhibited normal lipid profile and manifested oxidative stress status and noticeable inflammatory reaction in terms of positive C-reactive protein, increased ESR and increased lymphocyte count and noticeable thickened media-intima layer of aorta. In accordance to our finding, Ross (1999) indicated that inflammation has a pivotal role in the development of atherosclerosis. Moreover, a previous study suggested that measurement of the inflammatory marker C-reactive protein, may provide a useful method of assessing the risk of cardiovascular disease in apparently healthy persons, particularly when lipid levels are low (Ridker et al., 2000). In rats, CRP is not a typical acute- phase protein and exists in plasma under basal condition in a concentration which is 100 times higher than that in humans (Diaza Padilla et al., 2003).

In addition, it's suggested that the inflammatory effect of bacterial endotoxin induced oxidative stress which oxidizes LDL. Oxidized LDL, in turn, activates further inflammatory processes at the level of gene transcription such as up-regulation of nuclear factor kappa-B, expression of adhesion molecules, and recruitment of monocytes/macrophages and the generation of blood C-reactive protein (Ipatova et al., 2003). These activated macrophages produce numerous factors that are injurious to the endothelium (Kolodgie et al., 2003). Consequently, denudation of the overlying endothelium or rupture of the protective fibrous cap may result in exposure of the thrombogenic contents of the core of the plaque to the circulating blood and increased blood viscosity and coagulation (Cuthbertson & Christophi 2006). Moreover, it is likely that reactive oxygen species formation by phagocytes and subsequent modifications of vascular wall are involved in the early step of atherogenesis (Delbosc et al., 2002).

Observation that bacteremic rats exhibited structural abnormalities and considerable thickening of the aorta wall, nearly equals hyperlipidemia-bacteremia group might indicate that hyperlipidemia may have minor effect. Also, this might suggest that inflammation plays an essential role in the initiation and progression of
atherogenesis in presence or in absence of hyperlipidemia. Consistently, several studies indicated that inflammation causes abrasion of the overlying endothelium of the blood vessels through the exposure to the immune cells monocytes/macrophages and deposition of LDL-cholesterol leading to arteries stenosis even in normal lipid profile individuals (Delbosc et al., 2002).

4.3. Biochemical, hematological and histological effects of hyperlipidemia-bacteremia

It's worthy to note that the hyperlipidemic-bacteremic rats exhibited an additive effect regarding the oxidative stress parameters and the inflammatory reactions. This might indicate the pathophysiological effect is principally originated from the inflammation and that hyperlipidemia is a coordinator component. Alternatively, atherosclerosis could be recognized in part an inflammatory disease and that the lowering of lipid levels may represent an anti-inflammatory process. Consistently, oxidized low-density lipoprotein (LDL) cholesterol; infectious agents; toxins, the byproducts of cigarette smoking; hyperglycemia; and hyperhomocysteinemia are the probable causes of endothelial injury (Kesmarky et al., 2006).

5. Conclusion:

The study might indicate that there is a correlation between cholesterol level and heart disease but does not prove causation. Moreover, inflammation emerges to be independent risk factor for the development of atherosclerosis even in normal cholesterol level. In absence of the inflammatory reactions and oxidative stress, hyperlipidemia alone is not the principal risk factor for atherosclerosis.

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