Selenium supplementation protects from high fat diet-induced atherogenesis in rats: Role of mitogen stimulated lymphocytes and macrophage NO production

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The present study was designed to demonstrate the involvement of immune response in experimental atherogenesis. The mitogenic stimulation of lymphocytes and NO production by macrophages in experimental atherogenesis were studied. Further, influence of selenium a potent antioxidant was also studied in the disease process. Three different treatment groups of rats undertaken for study were: group I, control; group II, high fat diet (HFD) fed group and group III, HFD+Se supplemented group. Atherogenic conditions induced have already been explained earlier [Kang BPS et al. Gen Physiol Biophys, 17 (1998) 71]. Significant increase in 3H-thymidine incorporation was obtained in lymphocytes from HFD fed animals in both presence and absence of mitogen (Con-A). However, these values decreased in group III animals, which were supplemented with selenium. Similarly, NO levels with LPS+ and LPS' macrophages also found to be higher in HFD fed group and decreased in group III. These studies reveal the protective role of selenium in HFD-induced atherogenic process.

Involvement of macrophages, monocytes and lymphocytes in the immune system is well known. The role of these cells in the process of atherogenesis is well known. The role of these cells in the process of atherogenesis is also becoming evident since last decade. Macrophages have been demonstrated as a source of foam cells in the atherogenesis. Similarly, activated T-lymphocytes have been shown to be the onsets of atherosclerosis at all stages of development. Both helper (CD4+) and suppressor (CD8+) T-lymphocytes are reported to be present in the human lesions whereas in rabbit lesion CD4+ phenotype predominates. Immunocytochemical demonstration of MHC class II indicates that T-lymphocytes in lesions are immunologically tivated and are polyclonal in origin.

Stemme et al., in their studies have shown that the lymphocytes isolated from atherosclerotic plaque are immunospecific for oxidized LDL (ox-LDL), suggesting the activation of immune response due to increased lipid peroxidation products. Role of T-cells more important since it secretes INF-y, which can activate macrophages, resulting in inflammatory pones. Recently VLDL and LDL have been shown to induce nitric oxide (NO) synthesis, a duct of NO synthase enzyme, in macrophages. It is known to react with superoxide anion (O2-) to give peroxinitrite (ONOO-) which is a highly reactive free radical species and further induces ox-LDL.

Keeping these views in mind, the present study was undertaken to see NO production and immune status of atherogenic diet fed animals regarding mitogen (Con A)-induced stimulation index of lymphocytes as well as LPS-induced activation of macrophages in term of NO production. Further, Selenium (Se), a trace element and known antioxidant, has been used as a supplement along with HFD to see its influence on the proposed studies.

Chemicals
Cholesterol was obtained from LOBA Chemicals, Bombay, India. Sodium selenite and thiobarbituric acid were from Sigma Chemical Co. (St. Louis, USA). All other reagents and chemicals were obtained from SISCO Research Laboratories (India).

Treatment Protocol
Eighteen male Sprague-Dawley rats (100-125 g) were obtained from the Central Animal House, Panjab University Chandigarh (India), acclimatized to laboratory animals room and then randomly divided into three groups of six animals each: group I, control; group II, high fat diet (HFD), and group III, HFD+Se. Water was given ad libitum. The treatment protocol was followed for four months. Synthetic diet was prepared in the laboratory itself as
described by Abraham et al. The Se supplemented group of animals received 25 μg Se (equivalent to 1.0 ppm in diet) as Sodium Selenite/day/rat in solution form by oral intubation. It is well established that 0.02 ppm Se levels are adequate in mammals and levels at 2.0 ppm are considered subtoxic. The above dose was selected so as to have Se levels in excess but not toxic.

**Serum total cholesterol and triglycerides**
Serum total cholesterol was estimated according to the method of Chiamori and Henry. Triglycerides were quantitated in serum using enzokit supplied by Ranbaxy Diagnostic Ltd. (India).

**Mitogenic stimulation of lymphocytes**
Teased rat spleen lymphocytes cell suspension in HBSS from different groups was pelleted at 1000 rpm for 10 min and further washed twice with HBSS and finally loaded onto equal volume of Histopaque-1077 (Sigma Chemical Co, USA). Centrifugation was done at 1500 rpm for 20 min and buffy middle layer of lymphocytes were pelleted and washed twice with Dulbecco Modified Eagles Medium (DMEM). Finally washed pellet suspension was counted with hemocytometer using trypan blue dye (0.4%) and cell concentration was adjusted as per requirement.

Lymphocyte proliferation was determined in terms of 3H-thymidine (Bhabha Atomic Research Center; specific activity:18000 mCi/mM) uptake by DNA of replicating cells in culture and was quantitated as follows:

Cell suspension (100μl) was added to 96 well sterile tissue culture plate (Corning Inc., USA) to make 2x10^4 cells/well. To see the effect of mitogen, 5 ul of Con-A solution (1 μg/μl in DMEM) was added to each well. Finally DMEM was added to each well to make final well volume of 200 μl/well. Culture plate was incubated in 5% CO₂ environment in a humidified incubator at 37°C. After 48 hr of incubation, 1 μCi of 3H-thymidine was added to each well and culture plate was further incubated till the completion of 72 hr.

After incubation, cells from each plate were harvested onto glass wool filters using a semi-aumatic cell harvester. Filters were dried in oven at 37°C for 2 hr and then transferred to scintillation vials containing 7 ml scintillation fluid (3.0 g PPO and 1.5g POPOP in 1L of toluene). Counts were taken on liquid scintillation counter and DPM/well calculated for all the wells.

**LPS induced macrophages culture**
Macrophages from animals of all the three groups were isolated from peritoneal cavity by injecting cold Hank’s Balanced Salt Solution (HBSS), washed with DMEM, checked for cell viability and cells were counted on hemocytometer. Macrophages (2x10^5) were maintained for 24 hr in sterile 96 well tissue culture plates in DMEM with 10% fetal calf serum. Nitrite production was checked in supernatant of culture in presence and absence of lipopolysaccharide (LPS; 4 μg/well). Cell viability was also checked after 24 hr using MTT assay.

**Nitric oxide synthase (NOS) activity**
NO in terms of nitrite, a stable metabolic product of NO, was assayed in the plasma/macrophages using Griess reaction and citrulline (a stable by-product of the NOS-catalyzed reaction) was also measured in the plasma/macrophages according to the classical spectrophotometric protocol. Details of the procedure are already published by the authors in the previous publication.

**Statistics**
Data were expressed as mean±SE. Differences between groups were tested using Student’s t test for unpaired values.

A significant increase in 3H-thymidine incorporation was seen in the lymphocytes from the animals of group II in comparison to that of group I in both absence (P<0.05) and presence (P<0.001) of the mitogen, Con-A (Table 1). However, this change was highly significant (P<0.001) on addition of mitogen. Further, there was a significant decrease in the 3H-thymidine incorporation in lymphocytes , both without (P<0.01) or with (P<0.001) mitogen in animals of group III vis-à-vis group II animals (Table 1).

Nitrite levels in the plasma were seen to increase significantly (P<0.001) upon HFD feeding (Table 2) as compared to control group. However, these levels decreased significantly when Se was supplemented along with HFD (Table 2). Similar was the trend for plasma citrulline values where again its levels increased very significantly (P<0.001) in HFD fed group and decreased (P<0.001) in HFD+Se fed group. These results suggest increased NOS activity upon HFD feeding. Administration of Se along with HFD showed a significant decrease in NO levels in group III.

A significant increase (P<0.001) in NOS activity in terms of nitrite accumulation in the supernatant of the
macrophages in culture was observed in the group II as compared to group I (Table 2). Increase in the activity was observed both without and with LPS activation. These values decreased significantly (P<0.01) as compared to group II on Se supplementation (group II) in both, without and with LPS activation.

Present results showed an increased in vitro splenic lymphocytes proliferation in rats fed on HFD, both in presence and absence of mitogenic stimulation with con-A. Results depicting increased DNA synthesis on HFD feeding as studied by ³H-thymidine incorporation are in agreement with the studies of Ossman et al. Stemme et al. in their studies have shown that the T-lymphocytes isolated from atherosclerotic plaque are immunospecific for Ox-LDL suggesting the activation of immune response due to increased lipid peroxidation products.

Since oxidation of LDL represents a complex and heterogeneous series of reactions, it is likely that many structures are formed that may serve as neoantigens and give rise to a large variety of epitopes. Thus autoimmune response to Ox-LDL may be of more significant pathogenic importance in atherosclerosis.

Spleenic lymphocytes from Se-supplemented rats along with HFD showed an inhibitory effect on proliferation studies (in vitro) both in presence and absence of Con-A as mitogen. To the best of our knowledge, we did not find any report in literature regarding the role of Se in modulating immune response in HFD fed animals. Well-studied anticarcinogenic property of Se through inhibitory effect on cells growth form the basis for the supporting evidence for the present study.

Frankel et al. and Yan and Frankel have demonstrated that reaction products of selenite and sulfhydral compounds e.g. selenotrisulfide derivatives can inhibit purified DNA and RNA polymerases suggesting that these compounds could be the active species in the inhibitory effect of Se. In accordance with these views, flow cytometric studies done by Medina et al. indicated that selenite blocks cells in late S/early G2 phase of cell cycle. This fact very well substantiates the decreased proliferation of lymphocytes from group III rats of present study

Table 1—Mitogenic stimulation index of lymphocytes isolated from rat spleen after four months of diet feeding schedule.
[Values are mean±SE, figures in parentheses represent number of observations]

<table>
<thead>
<tr>
<th>Mitogenic stimulation index (DPM/2×10⁵ cells)</th>
<th>Control (Group I)</th>
<th>HFD fed (Group II)</th>
<th>HFD+ Se fed (Group III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Con A</td>
<td>308.0±37.4 (6)</td>
<td>1088.4±263.6 (6)</td>
<td>121.1±8.9 (6)</td>
</tr>
<tr>
<td>+Con A</td>
<td>8622±1245 (6)</td>
<td>57743±8999 (4)</td>
<td>19958±1836 (6)</td>
</tr>
</tbody>
</table>
HFD = high fat diet; DPM = disintegration per min
P values: *<0.05, **<0.01, ***<0.001 on comparison between group I and II.
P values: *<0.05, **<0.01, ***<0.001 on comparison between group II and III.

Table 2—Plasma nitrite and citrulline levels and nitrite accumulation by peritoneal macrophages (in presence or absence of LPS) from rats after four months of diet feeding schedule.
[Values represent mean±SE, figures in parentheses represent number of observations]

<table>
<thead>
<tr>
<th>Plasma nitrite levels (nmol/ml)</th>
<th>Control (Group I)</th>
<th>HFD fed (Group II)</th>
<th>HFD+Se fed (Group III)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.23±0.37 (7)</td>
<td>78.70±3.87 (7)</td>
<td>34.25±2.98 (7)</td>
</tr>
<tr>
<td>Plasma citrulline levels (nmol/ml)</td>
<td>1768±63 (7)</td>
<td>4478±161 (7)</td>
<td>2710±115 (7)</td>
</tr>
<tr>
<td>Nitrite accumulation by peritoneal macrophages (nmole nitrite accumulated/2.0×10⁸ cells)</td>
<td>LPS 7.39±0.357 (4)</td>
<td>13.55±0.13 (4)</td>
<td>10.48±0.61 (4)</td>
</tr>
<tr>
<td></td>
<td>LPS+Se 7.95±0.330 (4)</td>
<td>14.73±0.225 (4)</td>
<td>12.83±0.34 (4)</td>
</tr>
</tbody>
</table>
HFD = high fat diet
*values: *<0.05, **<0.01, ***<0.001 on comparison between group I and II.
*values: *<0.05, **<0.01, ***<0.001 on comparison between group II and III.
even after their stimulation in vitro.

Further, in the present study significant increase in plasma NO level was observed in 4 months HFD group and further on Se supplementation NO level decreased. Similar results were observed regarding NO level in LPS and LPS macrophages obtained from the HFD and HFD+Se supplemented groups. Stimulation over production of NO helps in production of peroxynitrite, which is involved in atherogenesis. The presence of iNOS and nitrotyrosines at particular sites such as macrophages, foam cells and smooth muscle cells further corroborates the suggested involvement of peroxynitrite in the oxidative modification of LDL.

Esaki et al. have shown the expression of iNOS in T-lymphocytes and macrophages of cholesterol fed rabbits. They have demonstrated that high cholesterol diet feeding can lead to stimulation of iNOS in foam cells present in atherogenic and macrophages through cell-cell interaction of both by a small amount of local cytokines by neighboring cells. Involvement of NO is also reported to be responsible for the induction of apoptosis in adrenal vascular endothelial cells. Moreover the studies showing involvement of NO to induce apoptosis in monocytes/macrophages indicate the negative effect of overproduction of NO as also seen in the present study on HFD feeding.

Plasma nitrite and citrulline levels as well as levels of nitrite accumulation by peritoneal macrophages (in presence and absence of LPS) decreased towards control levels when HFD was supplemented with Se. This depression in the NOS activity on Se supplementation seem to be mediated not by a single factor but by the sum total of various pathways where Se can interact directly or indirectly. However, the most plausible explanation still can only be in terms of antioxidant properties of Se.

As is evident from the values of nitrite accumulation by peritoneal macrophages in the present study, feeding of HFD led to the physiological condition which is actually inflammatory for macrophages as the NO production was showing trends in both with and without LPS i.e. in the absence or presence of macrophage stimulus under in vitro conditions. This provides the evidence that macrophages had probably got pre primed in vivo, so further availability of extraneous stimulus in in vitro resulted only in increase of the absolute values of nitrite accumulation without having any effect on the trends in different groups. Inflammatory response of the HFD is due to the enhanced lipid peroxidation associated with its feeding (as explained previously), which is reversible by Se supplementation.

Since iNOS contributes largely to the total nitrite levels in the present results, a control on iNOS by Se can ultimately lead to the decrease in plasma nitrite levels. Also, Southan et al. have shown the inhibitory action of Se compounds on iNOS and thus the possibility of oral administration of Se (as done in the present study) in inhibiting the iNOS activity cannot be ruled out. Furthermore, as Se supplementation resulted in upregulating the antioxidant enzymatic system of animals, free radical products of NO like peroxynitrite etc. can be more efficiently metabolized and finally leading to decrease in plasma nitrite levels.

On the whole, decreased NOS activity during Se supplementation along with HFD and the upregulation of antioxidant enzymatic process vis-à-vis corresponding decrease in lipid peroxidation make the situation favourable for pathogenesis of the disease. NO produced by cytokine-treated macrophages and hepatocytes plays a vital role in protective host responses to infectious pathogens. NO inhibits iron-sulfur-dependent enzyme involved in cellular respiration, energy production and reproduction. Toxic NO have been shown to be produced by cytokine-induced macrophages which have been implicated in cellular mechanism of nonspecific immunity to intracellular infection. These studies reveal the protective effect of dietary Se-supplementation on HFD-induced atherogenesis in rats.

References
NOTES


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