AMELIORATION OF CARRAGEENAN-INDUCED INFLAMMATION AND HYPOTHYROIDISM BY ETORICOXIB, A COX-2 INHIBITOR, IN RAT MODEL

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ABSTRACT
The present study was premeditated to reveal the amelioration of carrageenan-induced inflammation and hypothyroidism by Etoricoxib, a selective COX-2 inhibitor, in Wistar rat model. Carrageenan was administered to induce paw edema and alteration in thyroid functions, glucose metabolism; lipid peroxidation (LPO) in hepatic; renal and cardiac tissues along with serum total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels in female Wistar rats were studied. Besides tissue glutathione content, alteration in body weight along with daily rate of food and water intake was also analyzed. Carrageenan administration on the hind footpad of rats significantly increased paw volume, tissue LPO, serum insulin, glucose and TG levels; it lowered circulating HDL-C level with a marked decline in the serum levels of T₃ and T₄ and daily rate of food and water consumption. While etoricoxib administration along with carrageenan treatment; status of all the thyroid dependent parameters was reversed with a consistent increase in circulating thyroid hormone levels. The findings of present study are suggestive that the drug might be acting through the restoration of thyroid homeostasis which in turn ameliorates inflammation and oxidative stress.

Key words: lipid peroxidation (LPO); high-density lipoprotein cholesterol (HDL-C); total cholesterol (TC); thyroid hormones; oxidative stress.
1. INTRODUCTION

Inflammation is a natural defense of the tissues to protect against foreign substances or injury. It is a body's own reaction to assault an infectious cause or even just a physical; chemical; biological or traumatic damage [1,2]. Cyclooxygenase (COX) enzymes catalyze conversion of arachidonic acid to prostaglandin H2, serves as a precursor for the synthesis of prostaglandins, prostacyclins and thromboxanes. Two isoforms, COX-1 and COX-2, which are similar in properties but regulate the cellular expression differently [3,4] are expressed in various tissues. However, COX-2, being present at a basal level in certain tissues, is up-regulated in response to inflammatory stimuli [4,5]. In market, several drugs are available to manage inflammation and/or related disorders; nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly prescribed pharmacologically active compounds to treat chronic inflammatory conditions. While two main categories of NSAIDs; nonselective and selective, owe to their ability to inhibit specific type of COX expression [3,5]. Etoricoxib is a novel dipyridinyl, a selective COX-2 inhibitor, which has shown the efficacy similar to traditional NSAIDs in the rodent models of inflammation, pain and arthritis [6].

Various pharmacological active therapeutic agents are known to interfere with thyroid metabolism [7,8,9]. In the literature, reports are there suggesting that the patients particularly who are on chronic medications for curing other disease(s) develop thyroid abnormalities [10,11] as side effect. It is noteworthy that the patients suffering from inflammatory ailments are advised to consume medicines chronically. In literature, reports are available on thyroid inhibitory role of NSAIDs [10,11,12]. To date, to the best of our knowledge; there is no citation/report available on the influence of etoricoxib administration on thyroid homeostasis. It is well known that thyroid gland regulates almost all body functions including those of the antioxidant defense system, glucose and lipid metabolism [8,13,14,15,16]. Regardless of the above-mentioned pharmacological effects, no systematic study had been carried out in this direction with reference to etoricoxib.

Therefore, the present investigation was undertaken primarily to reveal the possible involvement of thyroid hormones in the mechanism of action of etoricoxib in the carrageenan-induced paw edema in rat model. Rat model of carrageenan-induced paw edema is a prolifically used animal model to evaluate anti-inflammatory activities of pharmacological agents [17]. Serum insulin, glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) levels and tissue (cardiac, hepatic and renal) lipid
peroxidation (LPO) along with reduced glutathione (GSH) content as well as alterations in body weight and daily rate of food and water consumptions, all being regulated by thyroid metabolism, have also been evaluated as supporting parameters [8,13,14,15,16].

**MATERIALS AND METHODS**

**Drugs and Chemicals**

The test drug etoricoxib (Arcoxia® Macleods Pharmaceuticals Ltd, Mumbai, India) was purchased from a registered local medical store, κ-carrageenan, 2-thiobarbituric acid (TBA), Ellman's reagent, sodium dodecyl sulphate, L-thyroxine (L-T4) and m-phosphoric acid obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Radioimmunoassay (RIA) kits, for the estimations of total T3 and T4 were procured from Bhabha Atomic Research Centre (BARC), Mumbai, India. All other chemicals were of reagent grade and obtained from Loba Chemie, Mumbai, India.

**Experimental animals**

Colony bred adult female Wistar rats, weighing 180-200 g were acclimated for a week before experimentation in a light (14 h light: 10 h dark cycle); temperature (23±2°C) and humidity controlled room with a free provision of laboratory feed (Gold Mohur feed, Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. Animals were maintained as per the guidelines laid down by the Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision on Experiments in Animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi. Institutional Animal Ethics Committee (IAEC) of Devi Ahilya University, Indore, India (No. Adm/Misc/2012/1483 dated 14.08.2012) approved the experimental protocol (No. IAEC 779 dated 24.07.2013).

**Study of paw edema**

The efficacy of etoricoxib in chronic inflammatory condition was evaluated in carrageenan-induced paw edema in rats, as described earlier [9,18]. Twenty-eight healthy female rats were divided into four groups (seven each). Animals of group 1, subcutaneously received the vehicle, normal saline (0.1 ml/animal) served as control, while those of groups 2, 3 and 4 were injected with 0.1 ml of 1% κ-carrageenan in normal saline into the right rear foot pad under light ether anesthesia for 21 days. The volume of injected paw was measured before (at 0 h) and after (at 3 h) the carrageenan administration using a Plethysmometer. Further a daily single dose of etoricoxib (3 mg/kg/day; group 3)[19] or vehicle (normal saline; group 2) was administered by gavage, 30 minutes before carrageenan administration for 21 days. Animals
in the group 4 subcutaneously received L-T<sub>4</sub> (10 µg/kg) [20], along with carrageenan for same duration. Drug or vehicle administration was done between 10:00 a.m. and 11:00 a.m. to avoid any circadian variation.

**Preparation of serum and tissue samples for biochemical analysis**

The experiment was terminated on 22<sup>nd</sup> day after the body weight of all the animals was recorded and then the over-night fasted animals were killed after exposing them to mild ether anesthesia. Blood sample from each animal was collected by cardiac puncture method and serum was isolated for carrying out various biochemical studies. After exsanguinations; the liver, heart and kidneys were dissected out, and washed with phosphate buffered saline (PBS, 0.1 M, pH 7.4) to get rid of blood clots; weighed and homogenized in a Remi Homogenizer to carry out tissue LPO, GSH and protein content.

**Radioimmunoassay (RIA) of thyroid hormones**

Total circulating T<sub>3</sub> and T<sub>4</sub> was estimated by RIA in serum samples following the standardized protocols, as routinely done in our laboratory [9,14,15,16]. In brief, the reaction mixture consisting of standard/sample, tris hydroxy-methyl amino methane buffer (0.14 M, containing 0.1 % gelatin; pH 8.6), radio labeled hormone (I<sup>125</sup>T<sub>4</sub> and I<sup>125</sup>T<sub>3</sub>) and the respective antibody was incubated at 37 °C (30 min. for T<sub>4</sub> and 45 min. for T<sub>3</sub>). Polyethylene glycol was used to terminate the incubation, followed by centrifugation at 2000 x g for 20 minutes. Finally the tubes were subjected to radioactivity counting for one minute (CPM) using an I<sup>125</sup> Gamma counter. Each assay was paralleled with a set of quality control sera of rat.

**Hormone assay of insulin**

Estimation of circulating insulin was done by RIA in serum following the protocol described earlier [8,13,14,15,16]. Briefly, 100 µl of sample/ standard was mixed with 200 µl of assay buffer, followed by the addition of 100 µl of primary antibodies (anti-porcine guinea pig IgG) and the mixture was incubated at 4°C for overnight. After incubation, 100 µl of radiolabelled (I<sup>125</sup>-labeled) insulin was added. To this 100 µl of secondary antibodies (anti-guinea pig rabbit IgG) were added after 3 hours of incubation at room temperature. Finally to each tube 1 ml of polyethylene glycol was added to stop the reaction. After gentle shaking, tubes were incubated for 20 minutes and centrifuged at 1500 x g for 20 minutes at room temperature. After decanting the supernatant tubes were subjected to radioactivity counting for one minute (CPM) using an I<sup>125</sup> Gamma counter.
Determination of lipid peroxidation (LPO)
Liver, heart and kidneys were homogenized in PBS (pH 7.4; 0.1 M) and the homogenates (10%) were centrifuged at 14000 x g for 30 minutes to obtain microsomal fraction. Briefly, LPO was determined by the reaction of 2-thiobarbutyric acid with malondialdehyde (MDA), a major end product of lipid peroxidation reactions, in acidic conditions. Tubes were incubated in a boiling water bath for 1 hr and absorbance at 532 nm was monitored on a Shimadzu UV-VIS 1700 Spectrophotometer [14,15,16,21].

Estimations of tissue protein and GSH
Total tissue protein concentration in homogenate was determined by the routine method of Lowery et al [22], using bovine serum albumin as standard. While tissue GSH content was measured by Ellman’s reagent method following the standard protocol [23], as earlier done in our laboratory [14,15,16].

Measurement of serum glucose and lipids
Estimation of fasting glucose in serum was done by enzymatic (glucose oxidase / peroxidase) method [14,15,16]. In brief, the protocol involves the formation of a red colored complex, when 4-aminoantipyrine and phenol react with glucose, as routinely done in our laboratory [14,15,16]. While serum TC and HDL-C was estimated, using spectrometric methods, as described elsewhere [24].

Statistical analyses
All the data are expressed as mean ± S.E.M. For statistical evaluation of the data, analysis of variance (ANOVA) and the Student’s t-test were used.

RESULTS
Carrageenan administration on the hind foot pad of rats increased the paw volume and the concentrations of serum insulin, glucose, TC and HDL-C (P < 0.001 for all) (Table-1); it decreased hepatic, renal and cardiac GSH contents (P < 0.001 for all) as well as daily rate of food and water consumption (P < 0.001 and P < 0.01, respectively) (Table 2). Administration of carrageenan on the hind foot pad of rats decreased circulating T₃ and T₄ levels (Fig. 1). Hepatic, renal and cardiac LPO (P < 0.001 for all) (Fig. 2) and body weight (P < 0.01) was also increased in response to carrageenan administration. Administration with etoricoxib to carrageenan treated animals reduced paw edema, hepatic; renal and cardiac LPO (P < 0.001 for all) and the concentrations of serum insulin, glucose and TC (P < 0.01 for all). It also
increased renal, hepatic and cardiac GSH contents ($P < 0.001$ all) and daily rate of food and water consumption ($P < 0.01$ for both). Carrageenan induced decrease in the concentrations of serum $T_3$, $T_4$ and HDL-C was reversed by the etoricoxib administration ($P < 0.001$ for all). Similarly, administration of L-T4 to carrageenan treated animals reversed most of the abnormalities produced by the irritant, however the effects were not highly pronounced as compared to carrageenan + etoricoxib group.

**Fig. 1**

![Graph showing changes in serum $T_3$ and $T_4$ concentrations](image)

**Fig. 1:** Effect of either etoricoxib or L-T4 administration on the changes in serum concentrations of $T_3$ and $T_4$ (ng/ml) in carrageenan (Carra)-induced paw edema in female rats. Each vertical bar represents the mean ± S.E.M. (n=7). $$***, P < 0.001$$ as compared to the respective control values and $$++, P < 0.001$$ as compared to the respective values of the carrageenan-treated group.

**Fig. 2**

![Graph showing changes in hepatic, renal and cardiac LPO](image)

**Fig. 2:** Effect of either etoricoxib or L-T4 administration on the changes in hepatic, renal and cardiac LPO (nM MDA formed / mg protein / hr) in carrageenan (Carra)-induced paw edema in female rats. Each vertical bar represents the mean ± S.E.M. (n=7). $$***, P < 0.001$$ as compared to the respective control values. $$++, P < 0.001; ++, P < 0.01$$ and $$+, P < 0.05$$ as compared to the respective values of the carrageenan-treated group.
Table 1: Effect of either Etoricoxib (3 mg/ kg, p.o.) or L-T4 (10 µg/kg, s.c.) administration on paw volume (% swelling), serum insulin (IU/L); glucose, total cholesterol (TC) and HDL-C (all in mg/dl) levels in carrageenan (0.1 ml/ animal) treated female rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Carrageenan</th>
<th>Carrageenan+Etoricoxib</th>
<th>Carrageenan+L-T4</th>
</tr>
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<tbody>
<tr>
<td>Paw volume</td>
<td>----</td>
<td>99.06***±5.11</td>
<td>52.10++±3.01</td>
<td>79.13+±3.51</td>
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<tr>
<td>Insulin</td>
<td>8.50±0.50</td>
<td>12.31***±0.70</td>
<td>9.01±0.61</td>
<td>10.03+±0.63</td>
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<td>Glucose</td>
<td>81.01±5.12</td>
<td>120.13***±7.12</td>
<td>91.02++±6.20</td>
<td>99.23+±6.10</td>
</tr>
<tr>
<td>TC</td>
<td>128.05±7.1</td>
<td>192.10***±12.81</td>
<td>132.81++±8.01</td>
<td>151.81+±9.12</td>
</tr>
<tr>
<td>HDL-C</td>
<td>41.02±2.23</td>
<td>24.16***±1.01</td>
<td>40.89++±2.08</td>
<td>30.98+±1.79</td>
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</tbody>
</table>

Data are mean ± S.E.M. (n=7). *** P < 0.001 as compared to the respective control values. ++ P < 0.001; ++ P < 0.01 and + P < 0.05 as compared to the respective values of the carrageenan-treated group.

Table 2: Effect of either Etoricoxib (3 mg/ kg, p.o.) or L-T4 (10 µg/kg, s.c.) administration on hepatic, renal and cardiac GSH content (µM GSH/mg protein); body weight (% increase) and daily rate of food (g/100 g body weight/day) and water consumption (ml/100 g body weight/day) in carrageenan (0.1 ml/ animal) treated female rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Carrageenan</th>
<th>Carrageenan+Etoricoxib</th>
<th>Carrageenan+L-T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic GSH</td>
<td>4.63±0.22</td>
<td>3.01***±0.20</td>
<td>5.62+++±0.27</td>
<td>3.78+±0.24</td>
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<tr>
<td>Renal GSH</td>
<td>3.89±0.17</td>
<td>2.60***±0.11</td>
<td>3.98+++±0.21</td>
<td>3.01±0.19</td>
</tr>
<tr>
<td>Cardiac GSH</td>
<td>3.16±0.16</td>
<td>2.01***±0.06</td>
<td>3.27+++±0.27</td>
<td>2.99+++±0.08</td>
</tr>
<tr>
<td>Body weight</td>
<td>8.04±0.41</td>
<td>10.98**±0.67</td>
<td>8.68+±0.51</td>
<td>9.01±0.64</td>
</tr>
<tr>
<td>Food consumption</td>
<td>7.97±0.53</td>
<td>5.05***±0.28</td>
<td>8.38±±0.47</td>
<td>6.11+±0.37</td>
</tr>
<tr>
<td>Water consumption</td>
<td>11.06±0.71</td>
<td>8.03**±0.45</td>
<td>10.88++±0.63</td>
<td>9.98+±10.58</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.M. (n=7). +++ P < 0.001 as compared to the respective control values. ++ P < 0.001; + P < 0.01 and + P < 0.05 as compared to the respective values of the carrageenan-treated group.

**DISCUSSION**

The results of the present study clearly demonstrate that carrageenan administration on the hind foot pad of rats increased paw volume, tissue LPO, serum fasting glucose, insulin and lipid concentrations with a decrease in T3, T4 and HDL-C levels, concomitant with the daily rate of food and water consumption indicating inflammatory, peroxidative, hyperglycemic...
and hypothyroid conditions. However, etoricoxib administration reversed the adverse effects produced by carrageenan including that of paw edema, altered glucose and lipid metabolism as well as tissue LPO and daily rate of food and water consumption with a consistent increase in circulating thyroid hormones. Interestingly, it is for the first time that we report the influence of etoricoxib on thyroid homeostasis and tissue LPO in an animal model of paw edema.

However, carrageenan-induced increase in the body weight and circulating lipid parameters, except HDL-C, suggestive of a hyperlipidemic state as previously observed [25] was also reversed by the etoricoxib administration. This is in accordance with the hypolipidemic effect of NSAIDs, as reported by others [26,27]. Interestingly, carrageenan-induced dyslipidemia seems to be an outcome of reduced activity of the enzyme- lecithin-cholesterol acyltransferase, which converts free cholesterol into cholesteryl esters [25]. Furthermore, carrageenan-induced hypercholesterolemia and increased body weight might also be due to the decreased level of circulating thyroid hormones; because hypothyroidism very often leads to obesity and hyperlipidemia [9,13,15,28,29]. Liver being the major site for cholesterol and triglyceride metabolism, and the thyroid hormones play an important part in hepatic lipid homeostasis. Thyroid hormones are reported to increase the expression of LDL receptors on the hepatocytes [30] and increase the activity of lipid lowering liver enzymes, hence resulting in a reduction in LDL-C levels. Thyroid hormones also increase the activity of apolipoprotein A₁, a major component of HDL-C [31]. However, following etoricoxib administration amelioration of dyslipidemia might be an outcome of drug-induced enhancement of thyroid functions, as thyroid hormones are known to be lipolytic in nature [8,13]. This fact is consolidated by the findings of the group who received both carrageenan and L-T₄. In the literature, there is no report available, till date, on the influence of etoricoxib on thyroid metabolism and tissue LPO. Therefore, observations made in the present study appear to be new and it adds to a better understanding of the impact of etoricoxib in inflammatory conditions.

Consistent with earlier reports [9,32] as carrageenan administration increased the concentrations of both serum insulin and glucose (a state of insulin resistance). The possible reason of altered glucose homeostasis could be the result of carrageenan-induced abnormalities including that of dyslipidemia, which sometimes, are reported to cause insulin resistance and hyperglycemia [33,34]. Administration of etoricoxib to carrageenan treated
animals reversed the hyperglycemia to normal. Since etoricoxib administration decreased the level of circulating lipids in carrageenan-treated animals, it is proposed that the reduction in insulin resistance might be an outcome of reduced level of various lipids as supported by earlier workers [8,13].

Carrageenan treatment to animals also resulted in the perturbation of hepatic, renal and cardiac tissues, as supported by increased LPO. Enhanced tissue LPO along with decreased GSH content in irritant-treated animals reflects the toxic nature of carrageenan in different tissues, as suggested recently by us [9] and others also [35]. Induced increased level of circulating lipids might have heightened the tissue LPO [36]. Nevertheless, another possibility of increased tissue LPO could be a secondary result of carrageenan-induced hyperglycemia, as sugars at higher concentration usually provides an oxidative environment [37]. Interestingly, in the present study carrageenan administration on the hind foot pad resulted into hypothyroidism. The hypothyroid nature of irritant was further supported by the observations made on food and water consumption, which were diminished in response to carrageenan treatment. Carrageenan-induced hypothyroidism appears to be an outcome of increased IL-1 level, which in turn decreases serum concentration of T₄ and T₃[38]. Since circulating T₃ is produced principally through the 5’ mono-deiodination of T₄ in the liver [39,40]. Interestingly, increased peroxidation of polyunsaturated fatty acids (PUFA) results in the deactivation of 5’mono-deiodinase enzyme activity leading to the reduced extra-thyroidal conversion of T₄ to T₃ [40,41]. Findings of the present study are in accordance to this fact, as etoricoxib administration to carrageenan treated animals resulted into amelioration of carrageenan-induced hypothyroidism with a concomitant decrease in the hepatic LPO. Later effect might be the result of etoricoxib-induced inhibition in IL-1 synthesis, as reported earlier for other NSAIDs [18,42]. Following etoricoxib administration, serum level of both the thyroid hormones was increased; it appear that etoricoxib regulates thyroid function both at the glandular level and at the level of peripheral conversion of T₄ to T₃ (mainly in hepatic and renal tissues). The restoration of euthyroid condition following etoricoxib treatment was further supported by decreased body weight of the animals who received both carrageenan and drug. The consistent increase in thyroid hormones might have diminished carrageenan-induced tissue LPO, as thyroid hormones have been reported to ameliorate oxidative stress [43,44].
Furthermore, the anti-inflammatory effect of etoricoxib might be an outcome of improved concentrations of circulating T₃ and T₄, as these hormones have been reported to possess anti-inflammatory activities [12,13]. This fact was supported by the finding made on animals who received both irritant and thyroid hormone, as exogenous T₄ administration decreased carrageenan-induced paw edema. Etoricoxib administration to carrageenan-treated animals was also found to be safe with respect to oxidative stress, as it did not induce tissue LPO, but fairly increased the GSH content in liver, heart, and kidneys. As all the mentioned parameters are dependent on thyroid function [13,14,15,16]. It is possible that etoricoxib might have brought these changes through the mitigation of carrageenan-induced hypothyroidism. This fact was further supported by the finding made on the group which received carrageenan and L-T₄ simultaneously, where exogenous administration of thyroid hormone ameliorated inflammation and oxidative stress [9,43].

CONCLUSION
The findings of the present study ravel the hitherto unknown influence of etoricoxib that the drug might be improving thyroid function at both the levels (glandular and peripheral) which in turn ameliorates inflammation and oxidative stress. It might be speculated that etoricoxib induced increase in HDL-C and decrease in LDL-C levels could be beneficial in reducing the onset of inflammation-induced cardiovascular ailments including atherosclerosis.

Declarations
Conflict of interest
The Author(s) declare(s) that they have no conflicts of interests.

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