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Cyclooxygenase-Independent Mechanism of Nonsteroidal Anti-Inflammatory Drugs

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ABSTRACT

A potential mechanism of NSAID-mediated anti-proliferative activity may be through the induction of NAG-1. The present study was conducted to investigate the possible role of selective and non-selective COX inhibitors in inflammation associated angiogenesis and apoptosis. Wistar rats were classified into 5 experimental groups; 9 rats each. Group (1) normal control and group (2) injected s.c. with 0.3 % carrageenan in muscle. Groups (3, 4 and 5) were injected s.c. with carrageenan and at the same time given orally 10 mg/Kg Celecoxib, 12.5 mg/Kg Nimesulide or 10 mg/Kg Sulindac, respectively. NAG-1 gene expression in the liver was measured by RT-PCR. Serum TNFα and muscle caspase-3 were measured by ELISA.Immunohistochemical detection of VEGF in the muscle was investigated. Carrageenan untreated rats showed insignificant change in NAG-1 gene expression compared with control group. Serum TNFα and muscle caspase-3 as well as VEGF expression in carrageenan untreated group were significantly increased compared with normal control rats. In Sulindac treated group, NAG-1 gene expression in the liver and muscle caspase-3 were significantly increased compared with Celecoxib and Nimesulide groups. TNFα serum level was significantly decreased in Nimesulide and Celecoxib treated groups compared with carrageenan and Sulindac groups. The examined NSAIDs proved proapoptotic and antiangiogenic effects.

Keywords: NSAIDs, NAG-1, Csapase-3,TNFα, carrageenan

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are heterogeneous class of drugs includes aspirin and several other selective COX 2 inhibitors (as celecoxib and nimesulide) or non-selective COX inhibitors (as sulindac) frequently used in the treatment of acute and chronic inflammation [1].In addition, the selective COX-2 inhibitors may help to prevent Alzheimer's disease, inhibit polyp formation, thus leading to a potential lesser risk of bowel cancer and reduce polyp formation in patients with familial adenomatous polyposis. NSAIDs have been proven to possess significant anti-proliferative potential in various cancer cells *in vitro* and *in vivo*[2].

Nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1) is highly induced by NSAIDs and numerous drugs and chemicals with anti-tumorigenic activities. NAG-1 plays significant roles in regulating proliferation and apoptotic activities[3]. A potential mechanism of NSAID-mediated metastasis suppression may be through the induction of NAG-1 as NAG-1–mediated decreased cell migration [4].

Apoptosis or programmed cell death is a normal component of the development and health of multicellular organisms and continues throughout adult life. In healthy organisms, apoptosis and cell proliferation are in balance [5].Two major general pathways of induction of apoptosis exist: the receptor or extrinsic pathway and the mitochondrial or intrinsic pathway that is produced following cellular stress. Both apoptotic signaling pathways converge at the level of the specific proteases; the caspases. Caspases are synthesized as pro-enzymes, which usually undergo proteolysis and activation by other caspases in a cascade. Functionally, three classes of caspases are distinguished; (i) the initiator caspases that are characterized by long prodomains (caspase-8, caspase-10, caspase-2 and caspase-9) (ii) the executioner or effector caspases containing short prodomains (caspase-3, caspase-6 and caspase-7) and (iii) the remaining caspases whose main role lies in cytokine maturation rather than apoptosis [5].Upon activation, initiator caspases cleave and activate effector caspases. Then, these cleave cellular substrates leading to all phenomena of the apoptotic morphology [6].

Cytokines are a diverse group of glycosylated proteins of particular importance in the control of cell proliferation, differentiation/activation and are crucial to host defense. Cytokines including interleukins, interferons and tumor necrosis factor (TNFα), which are produced by activated macrophages (7).Many inflammatory mediators have both direct and indirect angiogenic activities including; prostaglandins, TNFα, interleukins and nitric oxide [8].

Angiogenesis is a multistep process involving vessel sprouting, endothelial cell migration and

proliferation followed by capillary tube formation[9]. Angiogenesis and enhanced microvascular permeability are hallmarks of a large number of inflammatory diseases [10]. T cells can play a role in angiogenesis by delivering vascular endothelial growth factors (VEGF) to inflammatory sites(11).VEGF appears to be the most endothelial cell-specific and unequivocal angiogenic factor; it causes endothelial cell proliferation and migration and vascular permeability [8].

The present study was conducted to investigate the possible role of selective and nonselective COX inhibitors in inflammation associated angiogenesis and apoptosis.

MATERIAL AND METHODS

Forty five Wistar rats (male and female) weighing 120-160 g, were utilized in the present study. The rats were obtained from the animal house of Faculty of Pharmacy and Drug Manufacturing, Pharos University, Alexandria. The animals were maintained in plastic cages at 25°C in animal house for two weeks for acclimatization and were allowed free access to water and food. The rats were fed bread and milk.

Rats were classified into 5 experimental groups; 9 rats each. Group 1: normal control group given the vehicle (polyethelene glycol 400/saline 2:1 v/v, El-Amria and El-Nasr Companies). Group 2: inflammation control group injected s.c. with carrageenan (Sigma-Aldrich Inc. USA) 0.3 % in saline [12] on days 1, 4 and 7 [13]. Group 3: celecoxib group administered celecoxib (El-Amria Company) 10 mg/Kg bw orally daily [14]. Group 4: Nimesulide group administered Nimesulide (Cayman Chemical Co. USA) 12.5 mg/Kg bw orally daily [15].Group 5: Sulindac group administered Sulindac (Cayman Chemical Co. USA) 10 mg/Kg bw orally daily [16]. Rats of groups 3, 4, 5 were subjected to carrageenan injection as in group 2 on days 1, 4 and 7, whereas the administration of drugs continued from day 1 to day 7.

Twenty four hours after last treatment, blood sample was collected from retro orbital plexus of rats during anesthesia by diethyl ether (El-Gomhoria Chemical Company, Egypt). Blood sample was allowed to clot for 10 min at room temperature and then centrifuged for 10 min at 2000 rpm (800 g) (Baujahr centrifuge, Germany). The obtained serum was stored at -20°C (Deep freezer, Ilshin Lab Co., Ltd. Korea) until used for TNFα measurements by enzyme-linked immunosobent assay (ELISA)(17). Rats were sacrificed by cervical dislocation and then dissected. Livers were isolated and stored at -80°C (Deep freezer, Ilshin Lab Co., Ltd. Korea) for measurement of gene expression of NAG-1 by reverse transcriptase polymerase chain reaction (RT-PCR) [18].

The gastrocnemius muscle was divided into two portions. The first portion was kept at -80°C for measurement of caspase-3 by ELISA[19]. The second portion was embedded in 10% formaline (El-

Gomhoria Chemical Company, Egypt) and utilized for immunohistochemical investigations of VEGF [20].

Reverse transcriptase polyperase chain reaction (RT-PCR)

Total RNA was extracted from frozen muscle using Total RNA Extraction Kit (Bioer Technology, China). RNA (1µg) was reverse transcribed to give complementary DNA (cDNA) according to the manufacturer's instructions (The ProtoScript® AMV, First Strand cDNA Synthesis Kit, New England Biolabs, Inc.). cDNA was PCR amplified using 0.05 U/µL Taq DNA polymerase in a thermal cycler (Little Genius, Bioer, Germany). The primers for amplification of NAG-1 gene (Forward): 5′-AGCTGCTACTCCG CGTCAA-3′. **(**Reverse): 5′-GTAAGCGCAGTTCCAGCTG-**3′**.Initial pre-denaturation temperature was 94ºC for 1 min for one cycle. After that 35 cycles of the following program were carried out: denaturation step was at 94 ºC for 1 min, annealing step was at 58 ºC for 1 min and a final extension step was at 72 ºC for 1 min.

The PCR product obtained was then loaded onto 3 % agarose (Sigma-Aldrich Inc. USA) gel stained with ethidium bromide (Biobasic Inc. Canada) and the bands on the gel were visualized using UV transilluminator (Uvitec, EEC). The intensity of DNA bands were measured by photoshop version 7.

Measurement of TNFα and caspase-3 by ELISA

Rat serum was used for estimation of TNFα by ELISA using Rat TNFα assay Kit (BioVendor-LaboratorniMedicina). Citrate buffer (pH 5.5) was added to the muscle tissue (2:1) (v/w), which was then homogenized, then centrifuged for 10 min at 13,000 rpm (Baujahr centrifuge, Germany). The supernatant was used for estimation of caspase-3 by ELISA using Rat Caspase-3 ELISA kit (Cusabio Biotech CO., LTD).The concentration of caspase-3 in rat muscle was obtained from a pre-constructed standard curve and was expressed as ng/g tissue.

Immunohistochemistry for VEGF in rat muscle

R&D Systems' Cell and Tissue Staining Kits are intended for localization of VEGF. Detection is based on the formation of the avidin-biotin complex with primary antibody (VEGF specific IgG) that reacts with muscle VEGF. Sites of antigen localization were viewed using light microscopy. Expression of VEGF was evaluated as; negative expression (-), weak expression (+), moderate expression (++), strong expression (+++), intense expression (++++).

Statistical analysis:

Data were fed to the computer using the Predictive Analytics Software (PASW Statistics 18).

Quantitative data were described using mean and standard error. The comparison between two independent populations was done using independent t-test. Correlations between two quantitative variables were assessed using Pearson coefficient. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS AND DISCUSSION

The present study was conducted to investigate the possible role of selective and Non selective COX inhibitors in inflammation associated angiogenesis and apoptosis.

NAG-1 and COX-2 are involved in cellular processes such as inflammation, apoptosis and tumorigenesis. While COX-2 expression is highly induced in tumor tissue, NAG-1 expression is reduced [21].

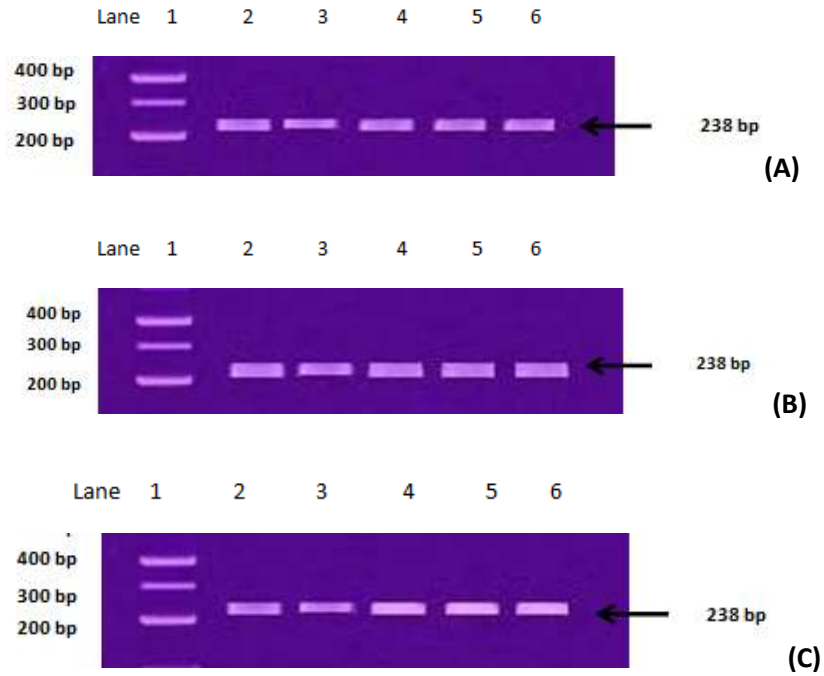


Figure (1): Ethidium bromide stained agarose gel showing bands of amplified PCR products of **NAG-1 gene** of rat liver. Lane 1: DNA marker, lane 2: carrageenan group, lane 3: normal control group, lanes 4, 5 and 6: NSAIDs-treated groups: **(A) Celecoxib** group, **(B) Nimesulide** group and **(C) Sulindac** group. Each lane represents pooled sample of livers of three different rats.

Effect of NSAIDs on gene expression of NAG-1 in rat liver

The RT-PCR products of amplified NAG-1 gene were separated by gel electrophoresis where the bands appeared at 238 bp (Figure1).The results showed an insignificant change in NAG-1

gene expression in liver of carrageenan treated rats compared with the normal control group. Celecoxib and Nimesulide treated rats showed insignificant change in NAG-1 gene expression compared with carrageenan group (Figure 2). NAG-1 gene expression was significantly increased after Sulindac treatment ($\simeq$ 18.7 % ↑, $p < 0.05$) compared with carrageenan group. NAG-1 gene expression showed significant increase in Sulindac treated group compared with Celecoxib and Nimesulide treated rats. Also, Sulindac group showed up to 1.2 fold increase in NAG-1 gene expression than the control group (Figure 2).Sulindac proved a potent effect as up regulator of NAG-1 in the liver. NAG-1 gene expression showed a significant increase in Sulindac group compared with other studied groups.

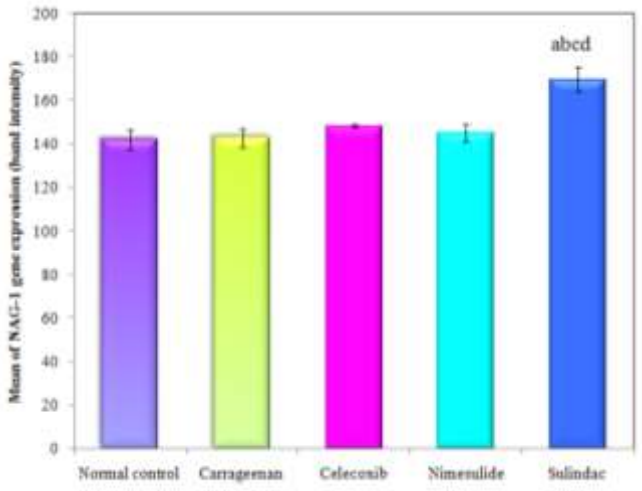


Figure 2 Effect of the selected NSAIDs on NAG-1 gene expression in the liver

Data represented as mean ± SEM; n= 9 for each group

a: significant *versus* normal control group

b: significant *versus* carrageenan group

c: significant *versus*celecoxib group

d: significant *versus*nimesulide group

The present results were in agreement with other research**,** that reported that NAG-1; a critical gene regulated by a number of COX inhibitors and has a chemopreventive effect on the development of human colorectal cancer and other cancers [22]**.** This suggestion was supported by the antitumor effects of NSAID are assumed to be due to the inhibition of COX activity, but COX-independent mechanisms may also play an important role. NAG-1/growth differentiation factor 15 (GDF15) was induced in the liver of Sulindac-fed mice and contributed to the prevention of tumor[23]**.**

Although Celecoxib in the present work exhibited insignificant effect on NAG-1 gene expression

in rat liver of carrageenan model of chronic inflammation, other investigators revealed that Celecoxib induced NAG-1 mRNA levels and apoptosis in patients with endometriosis [24], gastric cancer cells [25] and colon cells [26]. The latter effects of Celecoxib were dependent on drug concentration and duration of treatment [24].

The present results showed that Sulindac is the most NAG-1 inducer among the tested NSAIDs that is supported by other study that revealed Sulindac sulfide was the most significant NAG-1 inducer. NAG-1 is one of the genes responsible for growth suppression by Sulindac sulfide and provides a rationale for the chemopreventive activity of NSAIDs in ovarian cancer[27]. In another study, Sulindac treatment of mice increased the NAG-1 expression in the colon and liver, the gene which has proapoptotic and antitumorigenic activities that may contribute to cancer chemoprevention by NSAIDs[28].

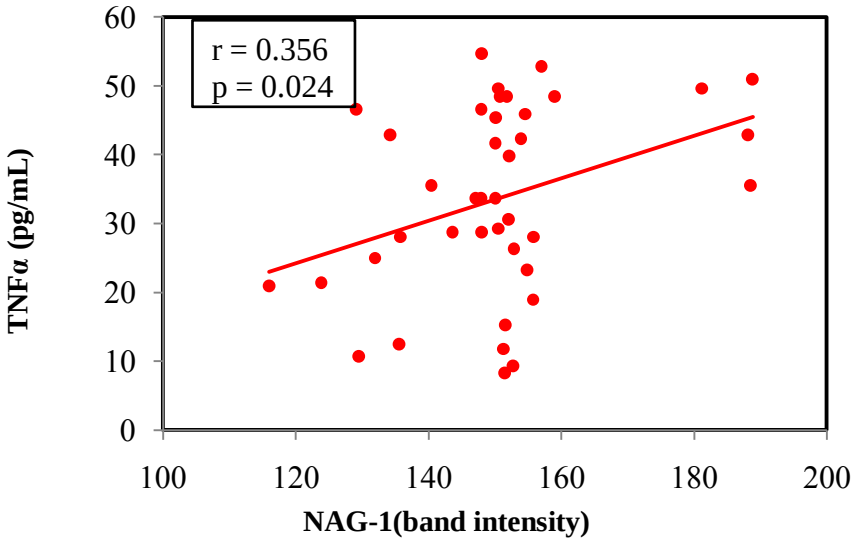


Figure 3: Correlation between gene expression of liver NAG-1 and serum TNFα

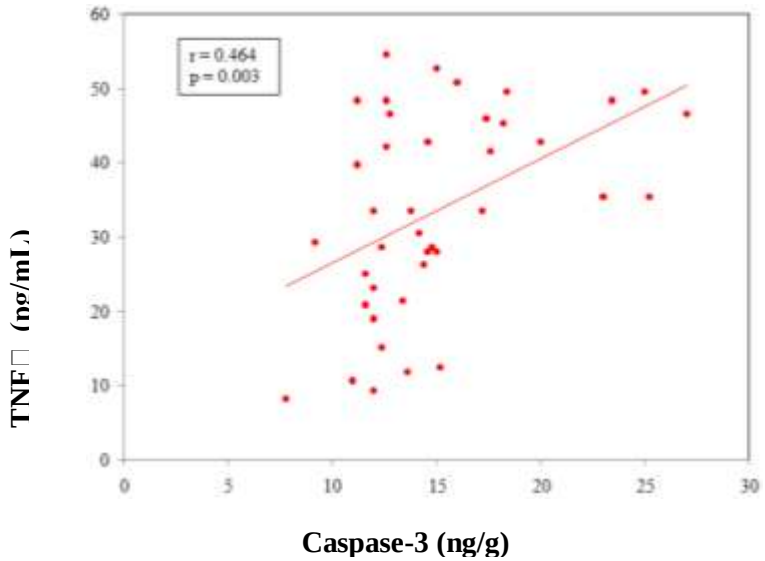


Figure 4: Correlation between muscle caspase-3 and serum TNFα

Effect of NSAIDs on caspase-3 level in rat muscle

Carrageenan treated group showed significant increase in caspase-3 level ($p < 0.01$) compared with normal control rats. Each of Celecoxib group and Nimesulide group showed significant decrease in caspase-3 level compared with carrageenan group ($\simeq$ 21.89 % ↓, $p < 0.05$) and ($\simeq$ 24.7 % ↓, $p< 0.03$), respectively. Caspase-3 level in Sulindac group showed significant increase compared with Nimesulide and Celecoxib groups. Sulindac group also showed up to 1.6 fold increase in caspase-3 level than control group (Table 1).

The present results showed that carrageenan caused significant increase in caspase-3 level in muscle tissue compared with corresponding control group. The increased level of caspase-3 observed in carrageenan group was significantly attenuated in both Celecoxib and Nimesulide-treated rats. In contrast, Sulindac produced an elevation of caspase-3 level, which was significant compared with the other experimental groups. These results provide a further support for the proapoptotic and antiproliferative effects of Sulindac.

The present results showed a significant positive correlation between serum level of TNFα and each of gene expression of NAG-1 in the liver($p = 0.024$, Figure 3) and muscle caspase-3 ($p = 0.003$, Figure 4), which were in accordance with studies, that found that carrageenan induces apoptosis, which is mainly due to up regulation of apoptotic genes such as TNFα, p53, caspase-8, caspase-3,as well as increases the level of active caspase-3[29].

Several reports suggested that NSAIDs induce cancer cell apoptosis and postulated that their cardiovascular toxicity may be due to endothelial cell apoptosis [30].

Celecoxib has been found to reduce the proliferation and induce apoptosis, of a variety of glioblastoma cell, which was mediated by enhanced caspase-3 and -8 activities [31].Also, the antitumorigenic effect of Nimesulide was found to be associated with a decrease in cell cycle regulatory proteins and the antiapoptotic protein $Bcl_2$and an increase in proapoptotic markers like caspase-3[32]. The inhibition of NF-kB by Sulindac was associated with greater caspase-3 and -9 activities, which may explain the molecular mechanisms underlying anti-tumor activities of Sulindac that include the induction of apoptosis in various cancer cells and the inhibition of malignant transformation[33].

NSAIDs induced apoptosis in a variety of cancer cells, including those of colon, prostate, breast and leukemia [34]. Cell biological studies provided circumstantial evidence that the mechanism by which these agents exert their antitumor effect should be attributed to induction of caspase genes transcription, resulting in apoptosis [35].

Effect of NSAIDs on TNFα level in rat serum

In carrageenan treated group, serum TNFα level showed a significant increase compared with the normal control group ($p < 0.05$)(Table 1).In Celecoxib treated rats, serum level of TNFα was significantly decreased($p < 0.05$) compared with carrageenan untreated group. Serum TNFα level in Celecoxib treated group was significantly decreased compared with that of Sulindac treated group (Table 1).In Nimesulide treated rats, serum TNFα level was significantly decreased compared with carrageenan group ($\simeq$ 39.4 % ↓, $p < 0.05$). Serum TNFα level in Nimesulide treated group showed a significant decrease compared with Celecoxib and Sulindac treated groups (Table 1).Serum TNFα level, although decreased by NSAID-treated groups *versus* carrageenan group, it remained greater than its corresponding value in the normal controls by up to 2.1-3.3 folds (Table 1).

Table 1: Serum TNFα and muscle caspase-3 in various studied groups

Groups	TNFα(pg/mL)	Caspase-3(ng/g)
Normal control	13.44± 1.61	11.84± 0.67
Carrageenan group	46.4^{a}± 1.42	18.04^{a}± 1.81
Celecoxib group	35.47^{b}± 3.12	14.09^{b}± 0.95
Nimesulide group	28.1^{bc}± 2.27	13.58^{b}± 0.41
Sulindac group	44.71^{cd}± 2.19	18.82^{acd}± 1.67

Data are presented as mean ± SEM; n= 9 for each group

a: Significant *versus* normal control group

b: Significant *versus* carrageenan group

c: Significant *versus* celecooxib group

d: Significant *versus* nimesulide group

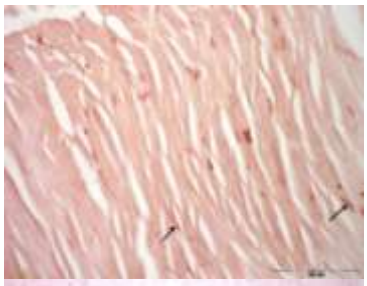
The present results revealed that injection of carrageenan into gastrocnemius muscle of rats resulted in significant increase in serum TNFα level compared with control group. Treatment with NSAIDs showed significant decrease in the serum level of TNFα compared with carrageenan treated group. However, Serum TNFα level in NSAID-treated rats didn't return to its normal value in control group. Nimesulide was most potent than other tested drugs in decreasing TNFα level in serum.

The elevation of serum TNFα level in carrageenan could be attributed to the fact that induces general inflammatory response mediated through the production of inflammatory cytokines. The local release of cytokines plays a major role in the sensory hypersensitivity associated with inflammation[36.] Treatment with Nimesulide and Celecoxib decreased the protein content of TNFα and monocyte chemoatractant protein-1, in adipose tissues of high-fat-induced obese rats[37].

In the present work, Sulindac did not affect serum level of TNFα in carrageenan-treated rats. These results could be interpreted by Sulindac or its metabolites enhances TNFα transcription or translation or interferes with TNFα clearance. TNFα augmented the cytotoxicity of Sulindac sulfide in primary hepatocytes thereby contributing to liver injury [38]. These findings suggest that enhancement of serum TNF-α concentration might be a common characteristic of drugs that induce idiosyncratic liver injury,and explain why Sulindac has been associated with a greater incidence of idiosyncratic hepatotoxicity in human patients than other NSAIDs [39].

Effect of NSAIDs on VEGF in rat muscle

Several diseases characterized by chronic inflammation are frequently associated with angiogenesis and fibrogenesis that account for the development of granulation tissue [40] Angiogenesis is reportedly enhanced by prostaglandins (PGs). COX-2 enhances neovascularization in tissue granuloma by PG-mediated expression of VEGF, and COX-2 inhibitors would facilitate the management of conditions involving angiogenesis [41].

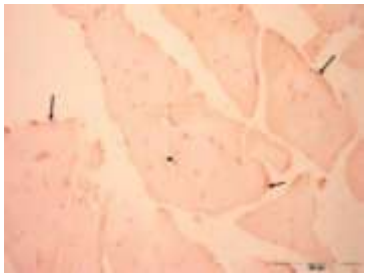


A: Rat muscle of normal control group showing moderate expression (++) of VEGF in endothelial cells of both blood vessels and capillaries associated with muscle fibers (X400).

B: Rat muscle of carrageenan group showing area of inflammatory muscle fibers, intense expression (++++) of VEGF granules concentrated in the cytoplasm of endothelial cells in most blood vessels and capillaries, congestion and dilatation of blood capillaries at the two sides of the figure (X100).

C: Rat muscle of Celecoxib group showing area of degenerative muscle fibers, dilatation of blood vessels and moderate expression (++) of VEGF in newly synthesized blood vessels (X400).

D: Rat muscle of Nimesulide group showing dilatation in blood vessels without congestion, degenerative muscle fibers and strong expression (+++) of VEGF in blood vessels and capillaries (X400).



F: Rat muscle of Sulindac group showing moderate expression (++) Of VEGF at the edge of each bundle and most blood capillaries (*) (X400).

Figure (5): Immunohistochemical detection of VEGF in rat muscle

Vascular endothelial growth factor (VEGF) was examined by immune histochemistry and was detected as brown granules in the cytoplasm of muscle fibers or precipitated on the endothelial cells of blood vessels. Normal control rats showed moderate expression of VEGF (++) in endothelial cells of both blood vessels and capillaries associated with muscle fibers (Figure 5A).Carrageenan-inflamed muscle showed intense expression (++++) of VEGF in blood vessels and small congested capillaries (Figure5B).

Celecoxib treated muscle showed area of degenerative muscle fibers, dilatation of blood vessels and moderate expression (++) of VEGF in newly synthesized blood vessels (Figure5C). Nimesulide treated muscle showed dilatation in blood vessels without congestion, degenerative muscle fibers and strong expression (+++) of VEGF in blood vessels and capillaries (Figure 5D). Sulindac treated muscle exhibited moderate expression (++) of VEGF in muscle fibers indicated regenerated muscle bundles and moderate expression of VEGF (++) in blood vessels (Figure5F).

In the present work rat muscle of carrageenan group showed intense expression of VEGF in blood vessels infiltrating muscle fibers. Treatment with Celecoxib and Sulindac decreased the expression of VEGF in blood vessels and hence reduced angiogenesis. Sulindac treated group showed expression of VEGF at the edge of muscle fibers indicating regenerated muscle bundles.

Inflammation and angiogenesis were demonstrated to be induced by injection of 0.03 % carrageenan into rat knees, which produced acute synovitis. Conversion of acute inflammation to chronic inflammation may be due to the stimulation of angiogenesis [42]. Our results could be supported by the reports of previous researches, which found that the microenvironment of dystrophic muscles is associated with variation in levels of markers of degeneration and regeneration, and that VEGF was measured as marker of regeneration. Muscle tissue repair is a complex biological process that crucially involves activation of stem cells. Satellite cells which are type of stem cells in the muscle tissue could excrete growth factors including VEGF that would induce angiogenesis and improve cell survival [43].

The beneficial effect of Celecoxib and COX-2 inhibitors [44] in retarding the angiogenic pathway by suppressing VEGF expression has been previously reported in prevention and treatment of

bone metastases [45]and treating diabetes-induced retinal abnormalities [46].

Angiogenesis is a key pathogenic event in hepatic fibrogenesis, which is mediated by activated hepatic stellate cells. Activated hepatic stellate cells produce COX-2 protein to induce VEGF production that participates in angiogenesis in injured liver. Pretreatment with COX-2 inhibitors, Nimesulide or indomethacin could significantly ameliorate the angiogenic event[47]. Also, sulindac sulfide significantly inhibited the production of VEGF *in vitro*, and significantly decreased the micro vessel density *in vivo*. Therefore, it was thought that angiogenic inhibition was important as one of the mechanisms of anti-tumor effect of NSAIDs[48].

CONCLUSION:

The examined NSAIDs proved proapoptotic and antiangiogenic effects. Sulindac exhibited the greatest effect as proapoptotic drug, which was mediated by upregulation of NAG-1 gene and induction of caspase-3. VEGF blockade was one of the mechanisms of anti-tumor effect of NSAIDs. VEGF blockade by Celecoxib represents a new therapeutic avenue in prevention of bone metastases and diabetes-induced retinal damage. Nimesulide was the most effective drug as TNFα inhibitor.

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