Chapter 1

Introduction

Prostaglandins (PGs), known to play important functions in bone repair and normal bone homeostasis, are synthesized by osteoblasts in responding to bone metabolism[1] and derived from arachidonic acid by cyclooxygenase enzymes, COX-1 and COX-2 in the process of inflammatory response[2]. Conventional NSAIDs inhibit the catalytic function of cyclooxygenase-1 and -2 (COX-1 and COX-2). Specific COX-2 inhibitors NSAIDs (specific COX-2), developed to avoid adverse effects from inhibition of the constitutive function of COX-1, is well received as an analgesic drug for management of chronic and acute pain[3,4]. COX-2 is inducible in responding to inflammation and stress suggesting an important role of COX-2 in the early stage of wound healing and repair[3]. Evidence from animal studies demonstrates inhibitory effects of specific COX-2 inhibitors on bone cell proliferation and delays fracture healing in dose and time dependent manner[5,6]. Essential roles of COX-2 in osteogenesis and fracture healing are clearly demonstrated[7,8]. The inhibitory effect *in vivo* studies was supported by *in vitro* studies that specific COX-2 inhibitors and conventional NSAIDs inhibit proliferation of human osteoblasts on the plastic surface of cell culture plate[9] and decrease differentiation of osteoblasts on rough surfaces of dental implants[10].

When specific COX-2 inhibitors NSAIDs inhibit the cyclooxygenase enzymes and the subsequent production of prostaglandins (PGs), they do not inhibit only the inflammatory process, but also inhibit production of PGs which are necessary for bone healing and metabolism. Although these adverse effects raise concern about the use of specific COX-2 inhibitors NSAIDs as an anti-inflammatory and analgesic drug in orthopedic procedures, clinical reports have been inconclusive concerning the effects of NSAIDs on bone healing. There is insufficient evidence to define effects of conventional and specific COX-2 inhibitors NSAIDs on osteointegration of implant prosthesis. Up to now, specific recommendation regarding the use of specific COX-2 inhibitors during osteointegration of dental implants can not be made. An association between exposure time dependent effects and therapeutic doses of specific COX-2 inhibitors NSAIDs on

osteogenesis and osteointegration of implant, requires further investigation to verify safe administration in chronic pain cases, such as osteoarthritis.

This study aimed to investigate the dose and exposure time dependent effects of specific COX-2 inhibitors on growth and differentiation of osteoblasts on smooth surfaces of titanium disks. It was hypothesized that specific COX-2 inhibitors decrease prostaglandin synthesis in a dose and time dependent causing decrease of growth and differentiation of osteoblasts on titanium surfaces.

Review of Literatures

Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are among the most widely used analgesic in the treatment of several forms of arthritis, menstrual pain and headaches. NSAIDs decrease inflammation by inhibiting the catalytic function of cyclooxygenase (COX) resulting in reduction of prostaglandin (PGs) and thromboxane A_2 (TXA_2).

Prostaglandin is produced from the release of free arachidonic acid (AA) from membrane phospholipids A_2 (PLA_2). The free AA converses to prostaglandin G_2 (PGG_2) by the cyclooxygenase activity, and then to prostaglandin H_2 (PGH_2) by its peroxidase activity. PGH_2 is the template leading to the formation of all PGs, including thromboxane A_2 (TXA_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin I_2 (PGI_2), and prostaglandin E_2 (PGE_2) by individual prostaglandin synthase enzymes (Fig.1).

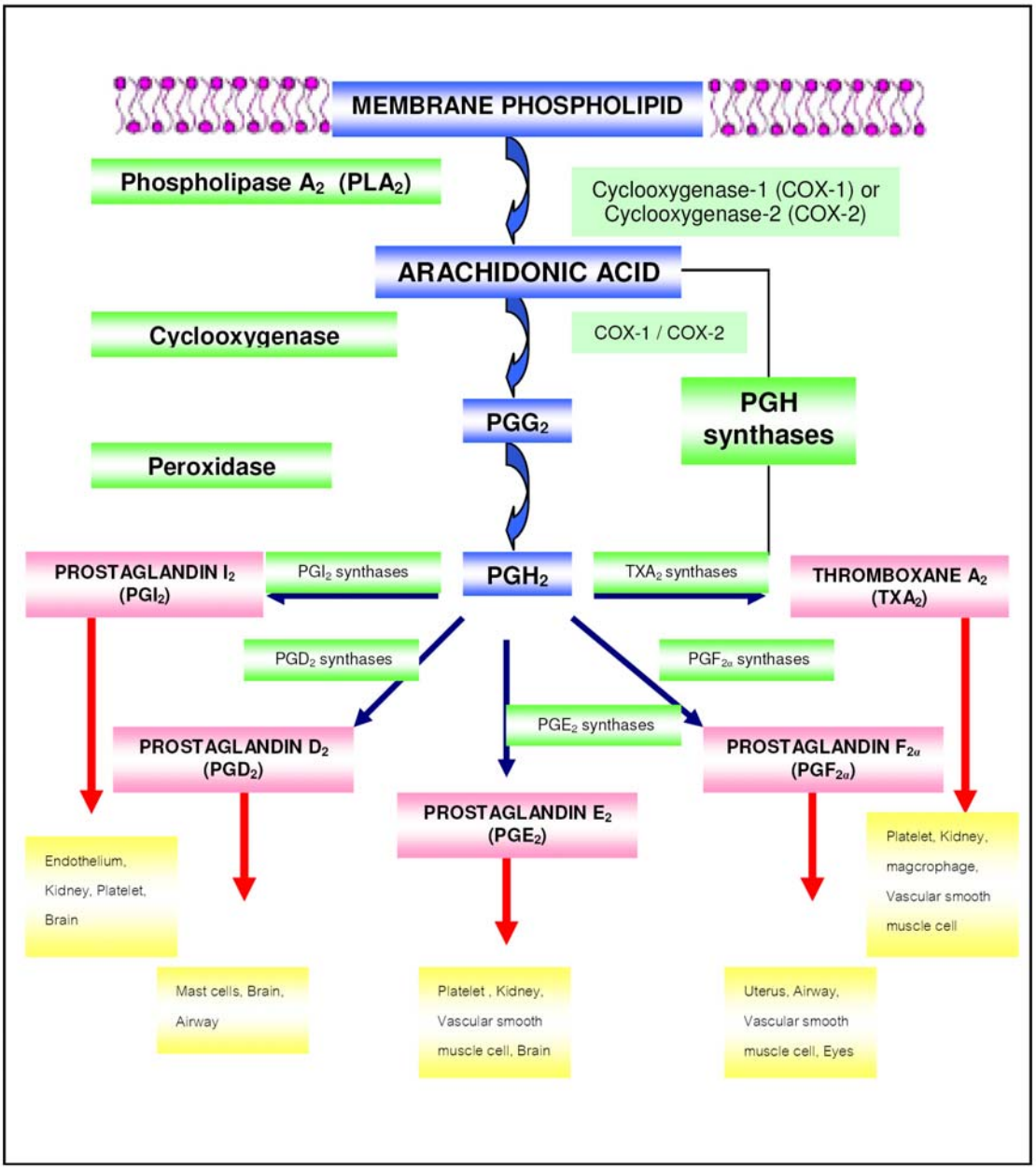


Fig. 1 Modified cyclooxygenase pathway shows prostaglandin synthase catalyzes the transformation of arachidonic acid (AA) into PGG_2, as well as the peroxidase activity that transform PGG_2 to PGH_2, the resource of substrate for the production of a variety of prostanoids[11]

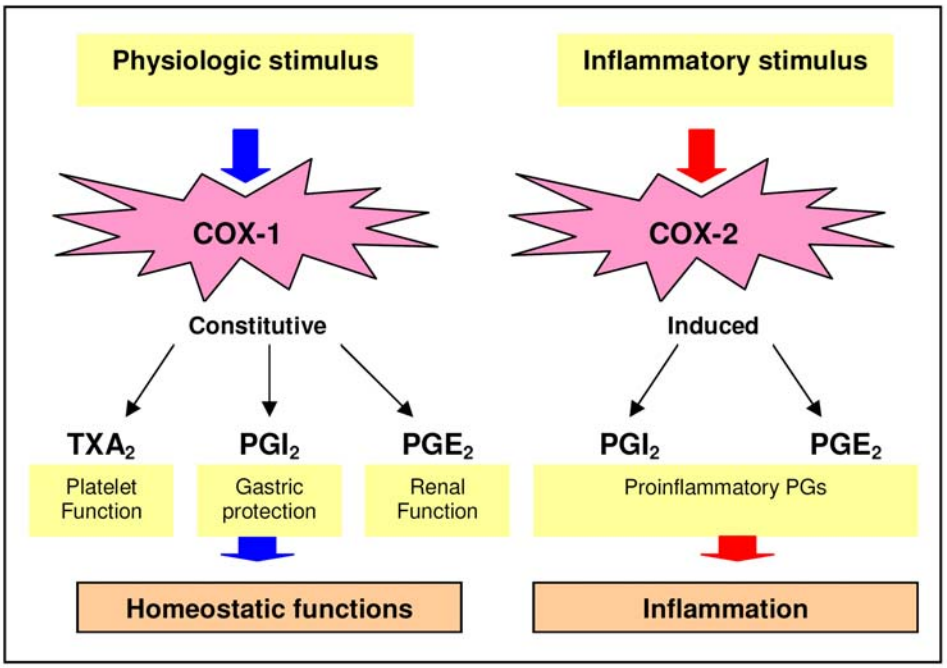


Fig. 2 Modified cyclooxygenase pathway shows COX-1, a constitutively expressed enzyme, and COX-2, an inducible enzyme. Both forms of COX regulate the synthesis of PGs[12]

NSAIDs such as aspirin, indomethacin, ibuprofen and ketorolac effectively suppress the inflammatory process through inhibition of cyclooxygenase enzymes and thereby reduce the production of inflammatory mediators such as PGs. Its ability to reduce inflammation is directly related to the ability to block PGs synthesis. NSAIDs are among the most commonly used medications for anti-inflammatory, antipyretic, chronic and acute pain relief such as arthritis, gout and ankylosing spondylitis. NSAIDs are effective against mild to moderate acute pain intensity, such as dental pain, and are also frequently used as an adjunctive medication in balanced anesthesia to reduce narcotic requirements. In addition, NSAIDs are extensively used by patients and physicians throughout the postoperative period to alleviate pain symptoms. It can be combined with opioid for management of severe pain when opioid doses can be reduced[13].

Another effect of NSAIDs is antipyretic since it can reduce body temperature in febrile states. Furthermore, some effects of PGs include delaying closure of the ductus arteriosus in premature infants. Therefore, the properties of blocking prostaglandins syntheses of NSAIDs are used for treatment to close the ductus arteriosus in neonates; however, it may cause premature contraction of the ductus in utero when administered in pregnant women[13]. Above all NSAIDs are also used for prevention of heterotopic ossification following post operative spinal fusion[13].

NSAIDs can be classified into two categories, chemical and pharmacokinetic classifications. The chemical classification is impractical in clinical application. Its subgroups are shown in Fig. 3 and Table 1. Pharmacokinetic classification, or half life of NSAIDs, is more applicable in clinical application and can be divided into three groups: short duration of action (half life < 8 hours), moderate duration of action (half life = 8-24 hours) and long duration of action (half life > 24 hours) (Table 2)[14].

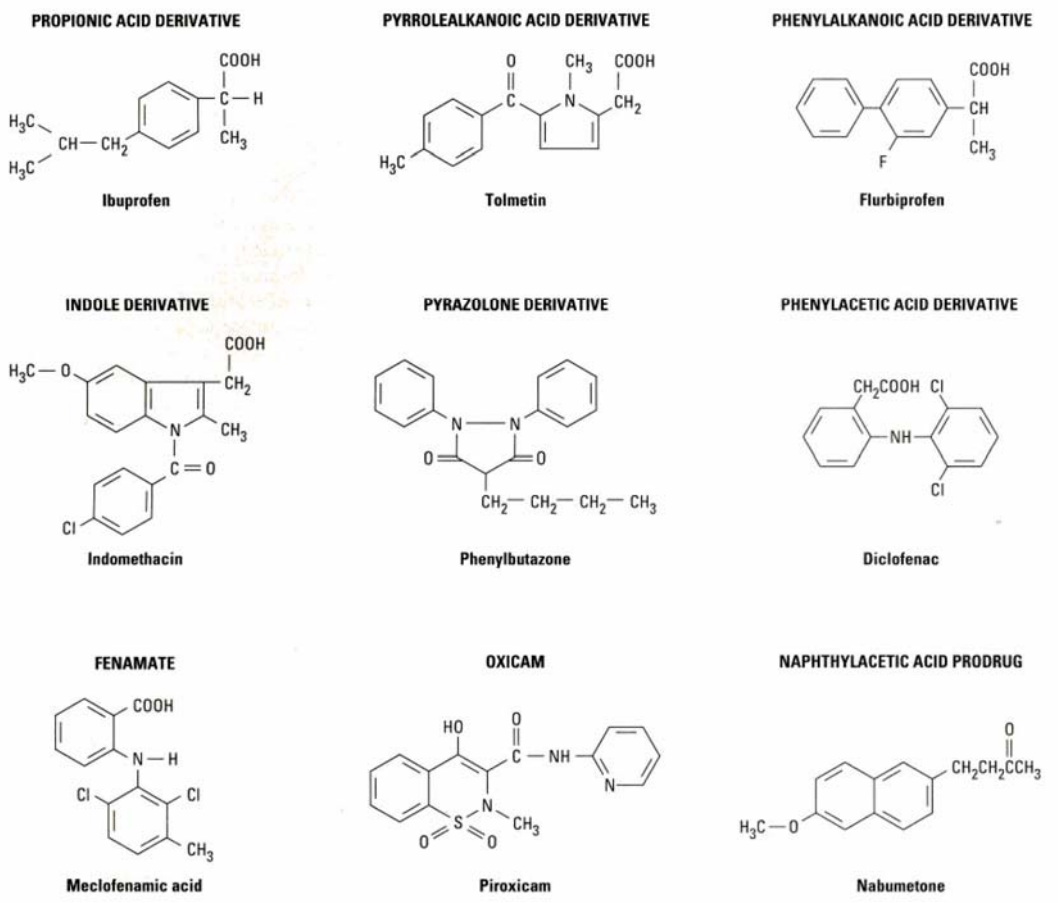


Fig. 3 Chemical subgroups of NSAIDs[14]

Table 1 The chemical classification of NSAIDs[15]

1. Acidic group
a. Carboxylic acids
i. Salicylic acids: aspirin, diflunisal, salicylsalicylic acid, salsalate, choline magnesiumtrisalicylate, sodium salicylate, salicylamide, benorylate
ii. Indole and indene acetic acids: indomethacin, sulindac, etodolac, proglumetacin, oxaprozin, tenidap
iii. Heteroaryl acetic acids: tolmetin, zomepirac, clopirac, diclofenac, alcofenac, ketorolac, fenclofenac, fentiazac, tromethamine
iv. Arylpropionic acids: ibuprofen, naproxen, flurbiprofen, ketoprofen, fenoprofen, fenbufen, pirprofen, loxoprofen, benoxaprofen, suprofen, indoprofen, oxaprozin, carprofen,tiaprofenic acid
v. Anthranilic acids: mefenamic acid, meclofenamate, flufenamic acid, niflumic acid
b. Enolic acids
i. Oxicams: piroxicam, piroxicam-β-cyclodextrin, meloxicam, tenoxicam, isoxicam, lornoxicam, sudoxicam, cinnoxicam, ampiroxicam, droxicam, pivoxicam, meloxicam, enoxicam
ii. Pyrozolones: phenylbutazone, azapropazone, metamizole, oxyphenbutazone, sulfinpyrazone, mepirizole, feprazone, monophenylbutazone, ketophenyl butazone
c. Sulfone component: nimesulide
2. Nonacidic group
a. neutral compounds: proquazone, fluproquazone, tinoridine, flinizole, bufexamac, tiaramide, epirazole
b. basic agents: benzyldamine, cloximate, difenpyramide
c. alkanones: nabumetone
3. Coxib group: celecoxib, rofecoxib, parecoxib, valdecoxib, etoricoxib

Table 2 The pharmacokinetics classification of NSAIDs[15]

NSAIDs	Daily Dosages
Short duration of action (half life < 8 hours)	**TID or QID**
Salicylate (low dose)	< 2500 mg
Ibuprofen	1200-2400 mg
Fenoprofen	1200-3200 mg
Ketoprofen	100-400 mg
Diclofenac sodium	75-150 mg
Flubiprofen	100-300 mg
Indomethacin	50-200 mg
Triaprofenic acid	400-600 mg
Meclofenanate	200-400 mg
Tolmetin	800-1600 mg
Moderate duration of action (half life = 8-24 hours)	**BID**
Salicylate (high dose)	3000-6000 mg
Fenbufen	600-1000 mg
Azapropazone	900-1800 mg
Naproxen	500-1000 mg
Difunisal	500-1000 mg
Sulindac	200-400 mg
Etodolac	600-1200 mg
Nimesulide	100-400 mg
Celecoxib	100-400 mg
Rofecoxib	12.5-50 mg
Long duration of action (half life >24 hours)	**OD**
Nabumetone	1000-2000 mg
Piroxicam	10-20 mg
Meloxicam	705-15 mg
Isoxicam	100-200 mg
Tenoxicam	10-20 mg
Phenybutazone	300-400 mg
Oxyphenbutazone	300-400 mg

Indomethacin, a conventional NSAIDs or non specific COX-1 and COX-2 inhibitors, introduced in 1963, is an indole derivative which is more effective than aspirin. In the laboratory, it is among the most potent inhibitors of prostaglandin synthesis. Indomethacin is useful in particular situations such as acute gouty arthritis, ankylosing spondilitis and is also

effective in extra-articular inflammatory conditions. In addition, indomethacin is used as an alternative drug for treatment of gout or when colchicines is unsuccessful or causes too much discomfort. For treatment of gout, 50 mg indomethacin is prescribed initially for three or four times a day or every 6 hours, and then reduced to 25 mg three or four times daily for about 5 days or until positive response occurs [2,13].

Disadvantage of non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are grouped in various chemical classes. This chemical diversity results in a broad range of pharmacokinetic characteristics. There are many differences in the kinetics of NSAIDs, but some general properties are still common. NSAIDs act by inhibiting cyclooxygenase (COX) enzymes which have two isoforms, COX-1 and COX-2. Both COX-1 and COX-2 catalyze the conversion of arachidonic acid to prostaglandins which pose various physiological effects including gastric protection, platelet function, and pain. Production of prostaglandins is blocked when COX-1 and COX-2 are inhibit, leading to both analgesia and causing gastrointestinal (GI) toxicity (bleeding, ulceration, and perforation of the stomach or large or small intestine). GI toxicity is a common and serious adverse effect of conventional NSAIDs.

Conventional NSAIDs are more potent in inhibiting COX-1 than COX-2, causing a decrease in constitutive prostaglandins catalyzed by COX-1. This effect leads to serious side effects, such as gastrointestinal bleeding and perforation and platelet dysfunction. NSAIDs affect hemostasis; for instance, a single low dose of 80 mg aspirin produces a slightly prolonged bleeding time, and it will be doubly prolonged if administration is continued for a week[13]. Selective COX-2 inhibitors have been developed to avoid those unfavorable effects by preserving the function of COX-1 and specifically blocking COX-2 functions[16].

Conventional NSAIDs induced gastrointestinal (GI) toxicity is a common and serious adverse effect. Symptomatic ulcers and ulcer complications associated with nonselective NSAIDs may occur approximately in 1% of patients treated for three to six months and up to 2-4% in patients treated for one year[17]. Therefore, duration of treatment is an important factor for selecting the type of NSAIDs. An ulcer is a breakage of the GI tract lining which extends to the submucosa or deeper. An ulcer may cause major upper GI bleeding, obstruction, or perforation. Gastric or duodenal ulcers found by endoscopy is approximately 15–30% in patients taking

NSAIDs regularly. Low-dose aspirin (325 mg daily) also significantly increases the risk of GI complications[18,19].

Conventional NSAIDs impair platelet aggregation by inhibiting COX-1, which is in platelets. However platelets do not contain COX-2, and clinical studies have shown that COX-2–selective NSAIDs do not affect platelet function at certain dosages[18-20]. Aspirin, a conventional NSAIDs often prescribed as a cardioprotective agent, irreversibly inhibits platelet aggregation from seven-to-ten-days of its lifespan, whereas other NSAIDs inhibit platelet action only for their half-life such as ibuprofen inhibiting for about two to five hours. Impaired platelet function is the inhibitory effect of NSAIDs on thromboxane, a potent aggregating agent, resulting in bleeding in many organs.

Most side effects of NSAIDs are unwanted. The discovery of a new class of NSAIDs, namely specific COX-2 inhibitors or coxibs, becomes important because it reduces GI toxicity induced by conventional NSAIDs. The clinical advantages of coxibs that are superior to conventional NSAIDs are related to the distinct roles played by cyclooxygenase-2 rather than cyclooxygenase-1[21].

As mentioned above, cyclooxygenase (COX) exists in 2 isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most tissue maintaining homeostasis levels of prostaglandin for normal function as a house keeping enzyme responsible for various physiological functions, which can be found in gastric mucosa, platelets, vascular endothelium and the kidney. Expression of COX-2 increases two to four fold increased following humoral stimulation[21].

The discovery of an isoenzyme, cyclooxygenase 2 (COX-2), in 1989 led to delineation of the distinct roles of COX-1 and COX-2. COX-2 shares 60% homology with COX-1 at the amino acid level and is not normally expressed in most tissue. It is generated in response to inflammation and inducible ten to eighteen fold by pro-inflammatory cytokines, growth factors, tumor promoters carcinogen, endotoxin, mechanical stress, injury and inflammation[12,16,22]

Recent studies described another isoenzyme, a cyclooxygenase 3 (COX-3), inhibited by acetaminophen[23], but one researcher supports this suggestion and so further studies are still needed.

Selectivity classification of NSAIDs are classified into 3 classes based on 3 important criteria: (1) IC_{50} of inhibition enzyme from recombinant enzyme model (2) ID_{50} of

inhibition enzyme from a test tube in gastric mucosa and a rat pouch model, and (3) the effect on COX-1 *in vitro* when a therapeutic dose is administered, demonstrated by: platelet function, a glomerular filtration rate and a gastrointestinal effect model. *In vitro* IC_{50} is shown in Table 3 and selectivity classification[24] is shown in Table 4.

Selective classification of NSAIDs

NSAIDs can be classified according to their selectivity toward functions of COX-1 and COX-2 enzymes into three subtypes: conventional NSAIDs, selective COX-2 inhibitors and specific COX-2 inhibitors, as described below (Table 4).

1. Classical or conventional NSAIDs

The IC_{50} of COX-2 more than IC_{50} of COX-1 means that more drugs are needed to inhibit COX-2 to achieve the therapeutic effects, meanwhile COX-1 is inhibited and affected more than COX-2.

2. Selective COX-2 inhibitors

The IC_{50} of COX-2 less than IC_{50} of COX-1; IC_{50} ratio of COX-2/COX-1 < 1 (not less than 0.01) or IC_{50} ratio of COX-1/COX-2 > 1 (not more than 100). However, when a high dose is administered, the therapeutic plasma concentration will be raised and results in COX-1 inhibition.

3. Specific COX-2 inhibitors

Specific COX-2 inhibitors is an NSAIDs that the IC_{50} of COX-2 is many times less than the IC_{50} of COX-1. When a therapeutic dose is administered, it does not inhibit COX-1. The calculated IC_{50} ratio of COX-2/COX-1 is less than 0.001 or the IC_{50} ratio of COX-1/COX-2 is more than 100.

Table 3 Modified comparison of NSAIDs for their selectivity towards COX-1 and COX-2 using *in vitro*[15]

NSAIDs	*In vitro* IC_{50} (μM)		
	COX-1 IC_{50}	COX-2 IC_{50}	**Ratio IC_{50}***
Classical type			
Naproxen	32	235	0.14
Piroxicam	662	679	0.97
Ibuprofen	38	117	0.32
Aspirin	145	180	0.81
Diclofenac	0.03	0.01	3
Indomethacin	0.1	1	0.1
Selective COX-2 inhibitors			
Etodolac	>100	54	>1.85
Nimesulide	50	9.4	5.32
Meloxicam	36	0.5	72
Specific COX-2 inhibitors			
Celecoxib	15	0.04	375
Rofecoxib	15	0.018	833

Ratio IC_{50} = Ratio IC_{50} of COX-1/COX-2

Table 4 The selectivity classification of NSAIDs from COX-2 concept[15]

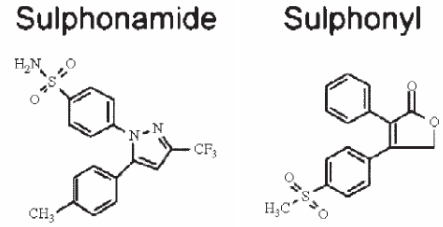
Class	Criteria	Drug
Classical NSAIDs	COX-2 IC_{50} > COX-1 IC_{50}	aspirin, indomethacin, ibuprofen, naproxen, ketoprofen, diclofenac, tiaprofenic acid, meclofenamate, piroxicam, tenoxicam, azapropazone, phenylbutazone, etc.
Selective COX-2 inhibitors	COX-2 IC_{50} < COX-1 IC_{50}	meloxicam, nimesulide, etodolac, carprofen, nabumetone
Specific COX-2 inhibitors	COX-2 IC_{50} << COX-1 IC_{50}	celecoxib, rofecoxib, parecoxib, valdecoxib, etoricoxib

Specific COX-2 inhibitors

Celecoxib and Rofecoxib (Fig.4) are the first generation of COX-2 inhibitors approved by FDA on 31 Dec 1998 and 21 May 1999 for treatment of chronic pain in rheumatoid arthritis (RA), osteoarthritis (OA), and acute pain relief associated with dental surgery and primary dysmenorrhea[25,26].

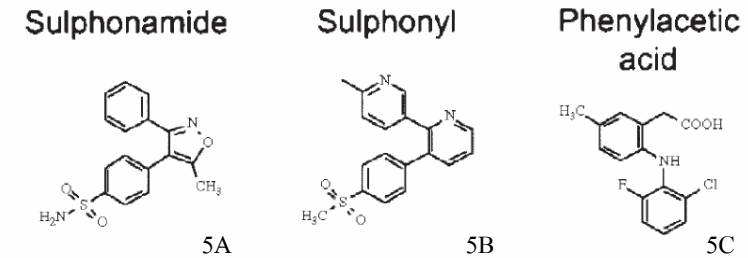
The second-generation of COX-2 inhibitors, with improved biochemical selectivity have been recently developed, such as etoricoxib, valdecoxib, parecoxib (valdecoxib injectable form) and lumiracoxib (Fig.5). They differ from the first generation on pharmacodynamic and pharmacokinetic features, therefore, their chemical efficacy also differ. However, their safety profiles are needed to be verified in comparative clinical trials. The potential use of highly selective COX-2 inhibitors at supratherapeutic doses is limited because it causes renal side-effects by dose - dependence. The improved GI safety profile of coxibs is the novel ability of selective COX-2 inhibitors versus nonselective NSAIDs[27].

Inhibition of COX-1 by celecoxib 800 mg/day, being 2-fold higher than the maximum chronic dose recommended in RA, may contribute to its failure in reducing the incidence of GI end-points in the CLASS study. The improved GI safety profile of the selective COX-2 inhibitors compared with nonselective NSAIDs was extrapolated from Pattrignani clinical study[27].



4A 4B

Fig. 4 First generation of COX-2 inhibitors celecoxib (4A) and rofecoxib (4B)



5A 5B 5C

Fig. 5 Second generation of COX-2 inhibitors valdecoxib (5A), parecoxib (5B) and lumiracoxib (5C)

Besides the GI safety profile, there are several advantages of COX-2 inhibitors that can be delineated as follows.

First, COX-2 inhibitors do not inhibit platelet function[13], therefore, there is no need to discontinue use of this drug before surgery. A previous study[28] showed that celecoxib 800 mg once daily and 600 mg twice daily (higher than the therapeutic dose) administered in normal volunteers for 7 days had no significant effect on platelet aggregation or bleeding time[28]. Administration of celecoxib or rofecoxib before surgery did not significantly increase intra operative bleeding.

Second, several lines of evidence suggest the critical role of COX-2 in tumorigenesis that blockneovascularization. COX-2 inhibitors may enhance basic fibroblast cell growth factor-induced angiogenesis through induction of vascular endothelial growth factors. In addition, celecoxib has been approved for the treatment of familiar adenomatous polyposis (FAP) by the FDA on the basis of its superiority to placebo in causing polyp regression[25,26].

Third, it has been shown that COX-2 inhibitors improve neurological disease by reducing dopaminergic neuron degeneration in animal models of Parkinson disease (PD), thus, it prevents the formation of the oxidant species dopamine-quinone involved in the pathogenesis of PD[27,29-31].

However, the renal effects of COX-2 inhibitors are similar to those of nonselective NSAIDs. They do not impair renal perfusion in healthy people, but they can lead to reversible renal ischemia and renal insufficiency in patients with congestive heart failure, cirrhosis, or volume depletion. Both NSAIDs and coxib may slightly elevate systolic blood pressure (average elevation is 3 to 5 mmHg, and possibly as much as 10 mmHg) in hypertensive patients treated with diuretics, β-blockers, or angiotensin-converting enzyme inhibitors[30]. Despite that coxib may impair normal bone healing in animal studies, there is no conclusion about the role of this drug in clinical application.

Table 5 General therapeutic dose of coxibs[17]

Drug	Condition	Doses
Celecoxib	Osteoarthritis	200 mg once daily or 100 mg twice daily
	Rheumatoid arthritis	100 to 200 mg twice daily
	Familial adenomatous polyposis	400 mg twice daily
	Acute pain and primary dysmenorrhea	400 mg initially, followed by an additional 200 mg if necessary on the first day, then 200 mg twice daily PRN
Rofecoxib	Osteoarthritis	12.5 to 25 mg once daily
	Rheumatoid arthritis	25 mg once daily
	Acute pain and primary dysmenorrhea	50 mg once daily PRN
Valdecoxib	Osteoarthritis	10 mg once daily
	Rheumatoid arthritis	10 mg once daily
	Primary dysmenorrhea	20 mg twice daily PRN
	Acute pain	40 mg initially, followed by 20 to 40 mg twice daily for 48 hours

NSAIDs and bone metabolism *in vitro* study

NSAIDs exhibit distinct inhibitory influences on osteogenic processes in various situations. They have been shown to influence bone metabolism, most likely due to the underlying inhibitory influence on prostaglandin production[2]. The majority of data implicate NSAIDs as detrimental agents to bone formation.

Ho and coworkers[32] evaluated the effects of NSAIDs (ketorolac and indomethacin) on proliferation and differentiation of osteoblast and found that the effect was mediated by the inhibition of PGs. Primary culture of parietal bone from fetal Spraque-Dawley rats were plating on culture plate 24 hr before supplementation with ketorolac, indomethacin, PGE_1 and PGE_2 in a serum free medium for 24 hours. They found that the NSAIDs inhibited osteoblastic proliferation in time (6 and 24 hours) and dose dependent manner. PGE_2 also demonstrated time and dose dependent effects in inhibition of DNA replication and cell mitosis over a concentration range of 0.1-1000 nM, but PGE_2 stimulated ALP activity and collagen type I on experimental day 10. It is hypothesized that PGE_2 may be involved in early stage of bone matrix maturation and subsequent bone mineralization. Therefore, NSAIDs affect osteoblast proliferation in an early stage of differentiation. However, stimulation effects of PGE_2 on ALP

activity and collagen type I could not explain its inhibitory effect on bone mineralization *in vivo*. They also demonstrated that after drug withdrawal, its effects may be reverted[32]. NSAIDs seem to delay the bone healing process and should be avoided immediately postoperation or should only be used in short-term administration, because early events in bone formation may be modulated by arachidonic acid metabolites[32].

After the work of Ho and coworkers[32], Chang and coworkers[33] investigated the inhibitory effect of NSAIDs on PGE_2 that affected osteoblast by the TGF-β1 pathway. In the same model they found that 24 hours treatment with NSAIDs (ketorolac and indomethacin) did not alter the bioactivity of TGF-β1 in osteoblasts, and supported the theory that NSAIDs inhibit PGE_2 effect on osteoblast but the inhibitory effects of PGE_2 may not be mediated by TGF-β_1.

Coetzee and coworkers[34] determined whether arachidonic acid (AA) alone or combined with estrogen and parathyroid hormone, induced PGE_2 synthesis in osteoblast-like cells and determined the level of PGE_2 mediated by COX-1 and COX-2. They used MG-63 and MCT3T-E1 in a culture system that was pre-incubated with indomethacin and NS-398 on culture plate. The result demonstrated that AA increased PGE_2 secretion in both cell lines. Both COX-1 and COX-2 affected the regulation of PGE_2 synthesis in MG-63. This study also showed that the inhibitory effect of NSAIDs (indomethacin) reduced the PGE_2 level rather than COX-2 inhibitors (NS-398).

NSAIDs and bone metabolism *in vivo* study

The majority of data implicate that NSAIDs are detrimental to bone formation. It is clearly demonstrated that NSAIDs inhibit bone formation and delay fracture healing in animal models[2,6,35,36].

Allen and coworkers[35] determined the effect of non-steroidal anti inflammatory agents, indomethacin and aspirin on healing fractures in a rat model. Two hundred and ten Spraque-Dawly rats were divided into 4 experimental groups and received therapeutic doses of aspirin 100, 200, 300 mg/kg/day and indomethacin 1, 2, 4 mg/kg/day for 21 days. The results demonstrated that fracture healing decreased in the group of aspirin 300 mg/kg/day and indomethacin 1, 2, 4 mg/kg/day. They revealed that the retarding effect of NSAIDs on fracture healing is a drug and dose related effect.

Keller and coworkers[37] studied the influence of indomethacin on regional blood flow, bone mineral content and mechanical strength of tibia osteotomy in rabbits. Thirty-eight Belgian rabbits received 50 mg of indomethacin as pre-medication and the administration was continued for 6 weeks over a post-operative period. At 2 weeks, the blood flow in a osteotomized leg of an experimental group was lower than the control group, but at 6 weeks it increased and higher than the control group. The bone mineral content in the indomethacin group was lower than the control. The authors postulated that NSAIDs inhibited the inflammatory process resulting in decreasing of regional blood flow into the repairing area and this condition led to a delay in bone healing. On the other hand it is implied that prostaglandins producing in the inflammatory process influence bone formation and increase fracture repair rates.

Trancik and coworkers[38] investigated therapeutic dose effects of indomethacin, ibuprofen and aspirin on bone growth into a porous-coated chrome-cobalt implant in a rabbit model. The animals were divided into 4 groups: (I) the control, (II) 1, 2, 3 mg/kg/day of indomethacin, (III) 17, and 34 mg/kg/day of aspirin, and (IV) 17 and 34 mg/kg/day of ibuprofen. Medications were administered subcutaneously 1 week preoperatively and continued until sacrification. The investigation was performed at 2, 4 and 8 weeks after implantation. Histological and histomorphometric analysis demonstrated that therapeutic doses of indomethacin (1, 2, 3 mg/kg/day) and ibuprofen (17, 34 mg/kg/day) and a high therapeutic dose of aspirin (34 mg/kg/day) caused reduction of bone ingrowth into a porous-coated implant. The authors concluded that indomethacin, aspirin and ibuprofen delayed bone ingrowths into a porous-coated implant in a dose dependent manner.

Engesaeter and coworkers[36] studied the inhibitory effect of indomethacin on fracture healing in a rat model. One hundred and thirty-five Wistar rats were divided into 2 groups according to medication administration methods, orally or applied locally on closed mid-diaphyseal fractures. Radiograph and fracture instability were tested at 5, 10 and 20 days of an investigation period. It was found that indomethacin administered orally and locally, inhibited bone repair.

Dimar and coworkers[39] determined deleterious effects of indomethacin on spinal fusion in a rat model. The study demonstrated that only 10% of an experimental group and 45% of a control group achieved a spinal fusion. Histological examination showed total resorption of bone graft and presence of giant cell surrounding a remnant of bone graft in the experimental

group, but in the control group osteogenesis and grafted bone were demonstrated in a fusion area. Results showed that therapeutic doses of indomethacin (3 mg/kg/day) administered immediately after surgery for 12 weeks delayed posterior spinal fusion and heterotopic bone formation in rats. It was postulated that delayed bone healing might be because of reduction of mesenchymal stem cell proliferation and inhibition of prostaglandin production.

It can be seen that NSAIDs inhibit fracture healing, osteotomy bone repair, bone growth in porous coated implants and spinal fusion surgery. These series of evidence suggest that NSAIDs may have detrimental effects on bone modeling and effect osteointegration on implant surfaces. Although NSAIDs have proven efficacy in the management of chronic and acute pain, this must be weighed against the risk of nonunion and its associated consequences about bone modeling and remodeling[39].

Specific COX-2 inhibitors and bone metabolism *in vitro* study

Evans and Butcher in 2004[9] compared the effect of classical NSAIDs, indomethacin and selective COX-2 inhibitors, DFU (5,5-dimethyl-3-(3 fluorophenyl)-4-(4 methylsulphonal) phenyl-2 (5H)-fluranone). Primary human osteoblasts were cultivated in culture medium supplementation with $3x10^{-7}$M of indomethacin and DFU for 5 days. It was found that indomethacin and DFU decreased numbers of osteoblasts and the effect was recoverable after the removal of indomethacin and DFU. It was concluded that specific COX-2 inhibitors and conventional NSAIDs have similar effects on the function of bone cells.

Boyan and coworkers[10] studied whether an increase in expression of the osteoblastic phenotypes of MG-63 cells on a rough titanium surface was mediated by COX-2. MG-63 osteoblast-like cells were seeded on three different titanium surfaces and tissue culture plate and cultivated in culture medium treated medications in an early stage of osteoblastic differentiation for 5 days after plating. It was found that $1x10^{-7}$M indomethacin, a non specific COX-1 and COX-2 inhibitors NSAIDs, inhibited the increase of PGE_2 production caused by surface roughtness by 80%. Reversatol, specific COX-1 inhibitors, inhibited up to 50% of PGE_2 production on cell culture plate and 80 % reduction of PGE_2 on the rough surface. In contrast, NS-398 had no effects on PGE_2 production on a smooth surface, but caused 60% decrease in PGE_2 on the rough surface. Indomethacin inhibited ALP on all surfaces to levels below basal levels, while reversatol and NS-398 had no effects on ALP activity. Regarding the effects of

NSAIDs to osteoblastic differentiation into a terminal stage, indomethacin and reversatol partially blocked stimulatory effects of surface roughness on osteocalcin production, but NS-398 completely inhibited effects of surface roughness on osteocalcin production. The study reported that both COX-1 and COX-2 regulated growth and differentiation of osteoblasts on the titanium surface of dental implant. The effects were varied upon characteristics of titanium surfaces. Specific COX-2 inhibitors demonstrated a greater inhibitory effect on prostaglandin and osteocalcin productions of osteoblasts on the rough titanium surface than non specific COX-1 and COX-2 and specific COX-1 inhibitors. The study proposed that COX-1 and COX-2 influenced response of osteoblasts to surface roughness in aspects of PGE_2, and osteocalcin productions and PGE_2 might be involved with expression of an ALP enzyme[10].

Specific COX-2 inhibitors and bone metabolism *in vivo* study

Simon and coworkers[6] assessed effects of specific COX-2 inhibitors on fracture healing of closed femur fractures stabilized with stainless steel pins in a rat study model. Rats were given with NSAIDs for 2 days before a closed femur fracture and after fracture for 8 weeks along the experimental period. The study was divided into 4 groups, 1 mg/kg of indomethacin, 4 mg/kg celecoxib and 3 mg/kg of rofecoxib, and a control. The results showed that indomethacin and specific COX-2 inhibitors, celcoxib and rofecoxib, delayed but did not inhibit fracture healing. The mechanical property result revealed that indomethacin and rofecoxib reduced mechanical stress, whereas celecoxib did not affect mechanical properties of fracture healing. Histological analysis, for indomethacin and COX-2 inhibitors showed abnormally formed cartilage within the callus. These observations were consistent with the radiographic and tortional mechanical testing data showing that celecoxib and rofecoxib could inhibit fracture healing. The results of COX-2 inhibitors presented a series of evidence in radiographic, histological, morphological, and biomechanical examination that it delayed fracture healing when administered before injury. These suggested that long term administration (8 weeks) of COX-2 inhibitors (celecoxib and rofecoxib) delayed fracture healing in rat experimental study[6].

Goodman and coworkers[5] examined the effects of a COX-1 and COX-2 inhibitors, versus specific COX-2 inhibitors NSAIDs on bone ingrowth and tissue differentiation in a titanium chamber implanted in the rabbit tibia. New Zealand white rabbits received naproxen sodium 110 mg/kg/day and rofecoxib 12.5 mg/day orally for 4 weeks. At 6 weeks after the

medication administration, immunohistochemistry and histomorphometric analysis of bone area were performed. The study reported that a non-specific COX-1 and COX-2 inhibitors, naproxen, and specific COX-2 inhibitors NSAIDs, and rofecoxib inhibited bone ingrowth and osteogenic differentiation in mice. This study clearly demonstrated that bone formation was suppressed by 4 weeks oral administration of COX-2 specific inhibitors. The authors suggested that long term administration of COX-2 inhibitors for arthritis and other conditions may potentially delay fracture healing and bone ingrowth[5].

Zhang and coworkers[8] examined the bone healing in wide type mice and mice genetically deficient for either COX-1 or COX-2 enzymes to elucidate roles of COX-1 and COX-2 in bone healing. The fracture healing model showed the critical role of COX-2 in fracture repair. Radiographs demonstrated delayed fracture healing of COX-2 knockout mice on fracture day 7, 14 and 21 days, which was different from COX-1 knockout mice and wide type. Histological analysis also supported radiographic results that COX-2 knockout mice revealed marked reduction of differentiation of mesenchymal stem cell into cartilage or bone in the intramedullary bone cavity. Large callus with devoid of osteoblast differentiation was found in COX-2 knockout mice and in situ hybridization performed in fracture callus on fracture day 14 found decreased expression of osteocalcin. COX-2 deficient mice demonstrated delayed fracture healing, delayed intramembranous bone formation and marked reduction of osteoblastogenesis of mesenchymal stem cells in bone marrow compared to COX-1 deficient and wide type mice. The authors concluded that COX-2 plays an essential role in endochondral and intramembranous bone formation during skeletal repair[8]. In addition, it was found that the defect in osteogenesis can be completely rescued by addition of PGE_2 to the culture medium, and that COX-2 was necessary in optimum osteogenic induction of BMP-2. It was postulated that prostaglandins were involved in recruitment and differentiation of osteoblasts[7,8].

Gerstenfeld and coworkers[40] measured the relative changes in the normal expression of COX-1 and COX-2 mRNAs over a 42 day period of fracture healing and compared the effects of commonly used non-specific NSAIDs, ketorolac, with selective COX-2 inhibitors NSAIDs: parecoxib, on fracture healing. Ketorolac and parecoxib in analgesic doses were administered orally. One hundred and twenty Sprague-Dawley rats were divided into 4 groups, Group I: control, Group II: 4 mg/kg of ketorolac, Group III: 0.3 mg/kg of parecoxib and Group IV: 1.5 mg/kg of parecoxib. Investigations were performed by examining plain radiographs,

histological sections and mechanical assessment of the callus formed at 3, 5, 7, 10, 14 and 21 days after fracture. They demonstrated that ketolorac and parecoxib delayed fracture healing. Bone healing in the non-selective COX-2 inhibitors group was worse than in the selective COX-2 inhibitors. In the control group, COX-1 mRNA levels were constant during the first 21 days, while COX-2 mRNA levels reached the peak of elevation during the first 14 days of healing and returned to a basal level after 21 days. These findings indicated that the osteogenic activity was active during the first two weeks of bone repair when high levels of COX-2 transcription and prostaglandin production presented[40].

Gurgel and coworkers[41] evaluated the dose and time dependent effect of selective COX-2 inhibitors (meloxicam) on critical size calvarial defect healing. Thirty-six Wistar rats were divided into 4 groups. Meloxicam 3 mg/kg was administered subcutaneously daily for 15 or 45 days after surgery. The results were compared with a control group of normal saline solution administered in the same fashion. Histological and histomorphometric results demonstrated that bone healing was delayed in the selective COX-2 inhibitors group when compared to the control group. They concluded that selective COX-2 inhibitors reduced bone healing in rats.

Murat and coworkers[42] studied effects of non-selective and high selective COX-2 NSAIDs on heterotopic ossification in rats. Heterotopic bone formation was observed in implantation of freeze-dried bone in muscle pouches of a rat model. The implants were removed and examined at 30 days after administration of 3 mg/kg/day of indomethacin (conventional NSAIDs) or 1.1 mg/kg/day of DFU (highly selective COX-2 inhibitors) or methyl cellulose as a control. There was no significant difference in the amount of bone formation among the highly selective COX-2 inhibitors, non-selective NSAIDs and the control[42].

Prostaglandin E_2 and Bone metabolism

Bone metabolism is regulated by multiple factors acting through osteoblasts and osteoclasts. Prostaglandins are synthesized by osteoblasts and act locally to stimulate both bone formation and resorption[2]. Adequate dosage of PGE_2 and mechanical loading are necessary to produce an anabolic response in the bone. PGE_2 adds bone to all bone enveloped by modelling and remodeling dependent the bone formation in a dose dependent manner[43]. PGE_2 induces bone formation by recruiting new osteoblast from bone marrow progenitors[44]. Positive effects of PGE_2 on the function of bone cells are demonstrated *in vitro* cell culture of fetal rat calvarial cells. It is

found that supplementation of low dosage of PGE_2, 3-30 nM, during post-confluence culture stage increases a number of mineralized nodules and levels of alkaline phophatase activity (ALP) and expression of osteopontin, but dose not effect the number of cells[1]. PGE_2, 10 nM – 10 μM, increases calcium ion concentration and stimulates type I collagen production[45]. Reher and coworkers[46] reported that ultrasound stimulated nitric oxide and PGE_2 production by human osteoblasts. Increase of osteoblasts growth by ultrasound stimulation was partly due to increase in the synthesis and secretion of PGE_2[47] (Fig.6).

Prostaglandins are abundant in skeletal tissue and are produced by osteoblasts and adjacent hematopoietic cells. The major effect of PGs *in vivo* is to increase bone turn over, i.e., stimulation of bone resorption and formation. Prostaglandins may mediate the effects of mechanism forces on bones and some of the changes that occur in bones with inflammation. Finally, prostaglandins may play a role in bone loss that occurs in menopause[21].

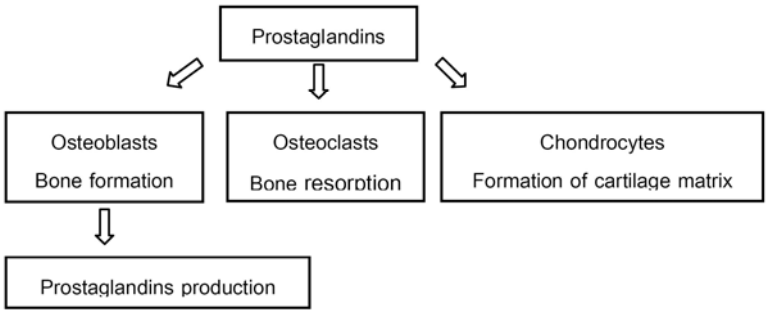


Fig. 6 Role of PGs on functions of bone cells (osteoblasts and osteoclasts) and cartilage cells (chondrocyte)[4]

Osteoblastic differentiation and expressions of osteoblastic phenotypes

The expression of osteoblastic phenotypes and differentiation genes are sequential, progressing from osteoprogenitor cells to preosteoblasts to osteoblasts and then to lining cells or osteocytes[48]. The temporal sequence is defined into three distinct periods: (i) growth and extracellular matrix biosynthesis; (ii) extracellular matrix development and maturation, and (iii) organization and extracellular matrix mineralization[49]. The differentiation state can be defined by detecting expressions of specific phenotypes and genes. During the early stage of differentiation, pre-osteoblasts express alkaline phosphatase (ALP) followed by secreting

type I collagen, then mature osteoblasts will be able to secrete osteocalcin and mineralized extracellular matrix[50].

ECM maturation begins with an increase in an expression of ALP which is found in preosteoblasts and osteoblasts. An ALP enzyme is expressed at the beginning of mineralization of an extracellular matrix and disappears when osteoblasts are embedded in the mineralized bone matrix to become an osteocyte. Detection of ALP activity is a common method for determining osteoblastic differentiation and mineralization status of the tissue[51]. It is regularly used *in vitro* as a relative marker of osteoblastic differentiation[50,52]. Mineralization of ECM concocts with the expression of osteocalcin and osteopontin mRNA. The expression of osteocalcin, an extracellular matrix protein, is closely related to mineralization of the extracellular matrix and is a marker of osteoblasts in a mature state[49,52].

Osteoclacin has 49 residue (5.8kDa) polypeptide which is highly conserved between species[53,54]. It contains γ -carboxyglutamic acid and has a high affinity for bone mineral. Osteoclacin is a vitamin K and vitamin D-dependent protein secreted by osteoblasts and is the most abundant and most widely studied of the non-collageneous proteins in bone. The majority of osteoclacin secreted by the osteoblasts is deposited in an extracellular bone matrix. It has long been accepted as an osteoblast specific product [53,54].

The temporal sequence of osteoblastic differentiation was started with formation of unmineralized nodules by day 5, and mineralized ECM on day 10. Nodules became larger on day 15 and were surrounded by numerous ALP-positive cells. ALP activity gradually increased by day 20. Parathyroid hormone responsiveness increased with time in the cultures. Osteoblasts produced no osteocalcin by day 10, but it was detected from day 15. The BMP-2 and BMP-4 mRNAs appeared in the cells forming nodules. These observations suggest that BMPs play an important role in the formation of mineralized bone nodules and confirmed that osteoblasts enzymatically isolated from newborn rat calvaria are a useful tool for studying the differentiation process of osteoblast[55].

Mouse calvarial cell line (MC3T3-E1)

The MC3T3-E1 pre-osteoblastic cell line is a spontaneously immortalized cell line of newborn mouse calvaria derived cells. MC3T3-E1 has been extensively used to study effects of various growth factors and hormone on differentiation of osteoblasts[52,56,57]. It is a well-

accepted model of osteogenesis *in vitro*[58]. Its ability to form well mineralized bone-like ECM containing markers of osteoblastic differentiation such as collagen type I, ALP and osteocalcin, makes this cell line an excellent model for studying osteoblastic differentiation. In addition, it has a distinct reproducibility advantage over primary cell culture systems[52,58].

MC3T3-E1 cells behave as immature, committed osteoblast cells, which continue to differentiate in response to intracellular and extracellular signals. Cells were recommended to be cultivated in α-modified Eagle's medium (MEM-α) and 10% fetal bovine serum, treated with ascorbic acid or other growth factors for special differentiations (i.e. β-glycerophosphate for bone nodule)[59]. Upon reaching confluence, this clonal cell line differentiates along osteoblastic lineage, sequentially expressing characteristic phenotypes of osteoblasts in a manner closely mimicking that of primary cultures of fractions 3-5 calvarial osteoblasts[59]. When cultured in the presence of ascorbic acid and β-glycerophosphate, MC3T3-E1 cells in postconfluent cultures will mineralize ECM accumulating hydroxyapatite[58].

MC3T3-E1 cells expressed osteoblastic phenotypes and mineralized ECM only after exposing to ascorbic acid. Ascobic acid supplemented in a culture medium is an essential factor controlling growth and differentiation of this cell line. It stimulates these cells to undergo a developmental sequence that includes the proliferation of undifferentiated precursors of osteoblasts, which subsequently differentiate into postmitotic osteoblasts being capable of expressing the osteogenic phenotypes. Mineralization is further stimulated by β-glycerophosphate. The expression of osteoblast markers follows a clear temporal sequence. The earliest effects of ascorbic acid are to stimulate type I procollagen mRNA and collagen synthesis (24 hours after ascorbate addition), followed by induction of ALP (48-72 hours) and osteocalcin mRNAs (96-144 hours). Actions of ascorbic acid on osteoblast markers gene expression are mediated by an increase in collagen synthesis and/or accumulation, because parallel dose-response relationships are obtained for ascorbic acid stimulation of collagen accumulation and ALP activity[59].

Growth and differentaition of MC3T3-E1

The MC3T3-E1 cell line has been widely used in the studies of proliferation and differentiation. It undergoes 3 stages of development: proliferation, extracellular matrix maturation, and extracellular matrix mineralization and then enters a period of apoptosis[60]. Sudo

et al.[58] investigated the capacity of a clonal osteogenic MC3T3-E1 cell line and found that the MC3T3-E1 cell line had high ALP activity in the confluent stage and was able to differentiate into osteoblasts and mineralize bone matrix *in vitro*. The cells in the growing stage showed a fibroblastic morphology and grew to form multiple layers. On days 21, clusters of cells exhibiting typical osteoblastic morphology were found in cell nodule regions. Such nodules increased in numbers and size with incubation time. These cells became easily identifiable with the naked eye by days 40-50. In the central part of well-developed nodules osteocytes were embedded in a heavily mineralized bone matrix while osteoblasts were arranged at the periphery of the bone nodules. Numerous matrix vesicles were scattered around the osteoblasts and young osteocytes[58].

Wang and coworkers found that matrix vesicles and plasma membranes of osteoblasts, young osteocytes, and lysosome-rich cells showed strong reaction to cytochemical staining for ALP activity and calcium ions. Minerals were initially localized in the matrix vesicles and then deposited on well-banded collagen fibrils. Deposited minerals consisted exclusively of calcium and phosphorus and some of the crystals had matured into hydroxyapatite crystals. The results indicated that MC3T3-E1 cells have the capacity to differentiate into mature osteoblasts and were able to mineralized extracellular matrix *in vitro*[61].

Wang *et al.*[52] isolated a series of subclonal MC3T3-E1 cell lines with high or low differentiation/mineralization potential after growth in the presence of ascorbic acid. Subclones were characterized by their ability to mineralize collagenous ECM *in vitro* and *in vivo* and expressed osteoblast-related genes. These subclones will be very useful for studying critical events in osteoblastic differentiation and mineralization.

MC3T3-E1 has been used for evaluating biocompatibility of various bone substitute materials and scaffold, such as calcium phosphate cement (CPC) and poly (lactide-co-glycolide) (PLGA) microspheres[62]. MC3T3-E1 cells were able to adhere, spread and proliferate on calcium phosphate-chitosan scaffolds (CPC-chitosan). The cells with sizes of 20–50 μm could infiltrate into the 165–271 μm macro-pores of the scaffolds[63]. Besides, when MC3T3-E1 cells were seeded on three-dimensional porous titanium scaffolds, it was found that differentiation of MC3T3-E1 was promoted through the addition of 50 μg/ml of ascorbic acid and 10 mM β-glycerophosphate into the growth medium [64].

Biocompatibility of titanium and osteoblast-titanium surface interaction.

In general, biocompatibility of metal is a function of their corrosion behavior, and for optimum effect, alloys need to be carefully tailored to minimize adverse reactions. Titanium and its alloys, notably Ti-6 Al-4V, are widely recognized as a good corrosion resistance metal, though there is evidence of titanium release into the tissues[65,66].

There is evidence that cell proliferation and differentiation as well as matrix production are altered by surface roughness, and cells at different stages of differentiation respond differently to the same surface. Hence the outcome of the interaction between artificial materials is probably recognized in a different way by cells, depending on the nature of the conformation. Interaction between cells and a biomaterial surface is expected to be influenced by the surface topography which indirectly influences the proliferation and differentiation of the cells[65].

In general, titanium alloys show good osseointegration. A titanium surface has a high affinity for bone cell attachment and osteoblasts are capable of laying down an extracellular matrix in direct contact with the surface oxide layer[67]. Titanium and its alloys generally exhibit excellent corrosion resistance, several authors indicate that the risk of local corrosion in tissue fluids can be excluded[68].

The extracellular matrix (ECM) formed by osteoblasts consists primarily of type I collagen and other ECM proteins such as fibronectin, laminin, vitronectin, osteopontin, and osteonectin. Pre-elaboration of osteoblast extracellular matrix altered the surface chemistry and microstructure of the composite biomaterial surface. This alteration is a cause of differences in cell-matrix adhesion and spreading. The changes in surface chemistry and microstructure may lead to the development of the cell-matrix interaction[69,70].

Surface roughness and composition of titanium implants currently used in dentistry and orthopedics are varied. Titanium alloys have virtually replaced titanium by itself because of the strength requirement of implant prosthesis. Both titanium and Ti-6Al-4V (Titanium alloy) develop a surface oxide layer due to the natural passiveness of titanium[71].

Response of osteoblast-like cells (MG63) to titanium and titanium alloy depends on surface roughness and composition of titanium[71]. The surface microarchitecture (smooth or roughness) can affect interaction between bone cells and the implant. Different contact areas and chemical composition affect bonding characteristics with bio-molecules which enhance the chemical stability/reactivity between cell and surface. Moreover, since osteoblasts are located on

the surface of a titanium implant, surface roughness affects the synthesis of biological factors by these cells and modulates the tissue response at the bone-implant interface[72-74]. Osteoblasts have initially a greater attachment to rough, sandblasted surfaces with irregular morphology[65,68,75,76]. Bone cells can regulate the orientation, mass and physical properties of the functional mechanical environment[77,78].

Martin, Kierwetter and coworkers classified surface roughness values (Ra) varying from <0.1 μm (smooth) to 3-4 μm (rough) to>6 μm (very rough). There are distinct differences in phenotypic expression. When surface roughness was increased, expression of osteoblastic phenotypes were increased, including increasing of alkaline phosphatase activity (ALP), osteoclacin production, collagen synthesis, proteoglycan sulfation, production of latent TGF-β and PGE_2, but reduced cell number and DNA synthesis (proliferation). The optimum surface at the Ra value around 4 μm appeared to affect response of osteoblasts. Cell proliferation was reduced but not blocked and phenotypic differentiation was enhanced. In contrast, cells on the smooth surface had high proliferation rates, but ALP and osteocalcin production were low[79]. Hence surface roughness of titanium affected proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63)[80]. Lincks and coworker[71] confirmed previous observations that osteoblastic like cells responded in a different manner to both surface roughness and material composition[71].

Microstructure of porous titanium scafflods promotes proliferation and differentiation of MC3T3-E1 in comparison to polished surfaces. This shows that average pore size of titanium scaffolds significantly affect only the proliferation stage, leaving the differentiation and subsequent mineralization processes unaffected. The microstructural properties of foam scaffolds showed slowing and shifted growth of cells to later stages of cell differentiation, toward mature osteoblasts[64].

Osseointegration

Osseointegration, or osteointegration, is a clinical term which refers to direct bone anchorage with an implant body. In consequence, asymptomatic rigid fixation is achieved and maintained in bone during long term functional loading to support prosthesis and transmit occlusal force directly to the bone[81]. Basing on a clinical point of view and undecalcified histologic tissue, Branemark et al, has used the term “osseointegration” to describe the

phenomenon of direct bone contact with a submerged titanium implant. This evidence was first published in 1969, and defined as a "direct structural and functional connection between the organized vital bone and the surface of a titanium implant, capable of bearing the functional load". Since then, the terms osseointegration and functional ankylosis have been used widely but many clinicians and reserchers still use the term of osteointegration[82].

The primary osteointegration is mediated by an oxide surface of 2.0-5.0 nm, which covers the titanium and is mainly composed of titanium dioxide. Some evidence supports the hypothesis that the interface is strongly influenced by biomechanical factors, which have more influence on bone regeneration than the biomaterial properties[83].

There are many factors influencing the success of osteointegration. Osteointegration involves a complex series of events that are tied to discrete phases of osteogenesis including osteodifferentiation, matrix production, and mineralization, followed by remodeling[84].

Implant success can be achieved primarily by enhancing osteointegration. When an endosteal implant is inserted, it causes tissue injury and then induces an expected slight inflammatory reaction. A cellular response induced pluripotential cells to differentiate into a variety of cell types required for healing. Within 48 hours, a clot is organized, and the fibroblasts begin to lay down threadlike collagen fibers. At the moment, blood borne cells continue to lyses and remove debris, then osseous healing begins. Osseous healing around dental implants can be simply classified in to four stages as follows[85].

- ***Stage One:*** The vascular Sprouting Stage occurs 3 to 7 days following implantation. It is the earliest angiogenic and osteogenic phase. This early evidence of angiogenesis is located in the walls of the prepared osteotomy. This could be observed in the vascular sprouting from immature sinusoidal capillaries invading the granulation tissue. After the first week, the osteotomy site is rapidly filled with fine collagen fibers and fibroblasts, or undifferentiated mesenchyme.
- ***Stage Two:*** At the Early Bone Formation Stage, bone formation period occurs 2 weeks after implantation. It is the initial osteogenic stage and corresponds to the formation of bone trabeculae. Again, vascular ingrowth precedes rapid osteogenesis. Ridge like bone with sinusoidal capillaries filling grooves is observed. Discontinuous bone segments at the base adhere with a basketlike capillary network and develop into continuous new bone. The initial osteogenic unit is composed of one sinusoidal capillary and its first new

bone segment. In some cases, a thin fibrous connective tissue appears between the interface and the new bone, even as late as 2 weeks following implantation.

- ***Stage Three:*** The Bone Growth Stage occurs 4 weeks following implantation. The initial primary spongiosa transforms to secondary spongiosa and proliferates to form new alveolar bone.
- ***Stage Four:*** The Bone Maturation Stage period extends from 6 to 8 weeks following implantation. At this time, the formation of bone around the implant is nearly complete. A capillary plexus is between the original bone bordering the osteotomy and the new bone bordering the implant interface. At porous interfaces, it has been reported that pores of implant surface larger than 100 nm in diameter can accommodate bone ingrowth. Additional healing time may be required in such individualcases[85].

Relations between inflammatory joint diseases and dental implant

Rheumatoid arthritis is a chronic autoimmune disease characterized by inflammation of multiple joints with proliferation of the synovium and progressive erosion of the cartilage and bone, which often occurs symmetrically on both sides of the body. It's a common discomfort that is found in 1-2% of the Asian population and in females more than males in a ratio of 3:1. It normally occurs earlier in life than osteoarthritis and patients are often in their 30s and 40s[86].

Osteoarthritis is known as degenerative joint disease. Pain caused by osteoarthritis usually begins in gradual stages and progresses slowly over many years. Osteoarthritis is not life threatening but the quality of life can significantly deteriorate due to pain and loss of mobility. No treatment can cure OA. Specific COX-2 inhibitors are prescribed to reduce pain and decreases patient discomfort[87].

A clinical audit of Australian healthcare in 2001 was performed on 1417 patients with an age range of 15-98 years. Thirty six percent of patients were over the age of 70 years and 569 patients in 1417 cases (40%) were taking celecoxib. Osteoarthritis was the most common indication for COX-2 therapy (68%)[88]. In Spain, celecoxib was the fifth most frequently prescribed NSAIDs for specialty physicians[89]. Prevalence of osteoarthritis has increased mainly due to increasing life spans, and the consequences have a significant impact on society, because osteoarthritis is a cause of chronic disability at old age.

In the USA, more than 13% of osteoarthritis patients have an age range of 55-64 years and more than 17% are aged between 65-74 years old. The prevalence of OA increases substantially after age 40 in women and 50 in men[90].

A clinical audit in Thailand between October 1997 to September 1999 conducted at Siriraj hospital found knee osteoarthritis in 392 patients, with a male to female ratio of 1:3.6 the mean age being 67.7±6 years old[86]. The survey correspondes with a report of Charoencholvanich and Pongcharoen where the mean age of osteoarthritis patients was 63 years old and the majority of patients were female being 86% of the total osteoarthritis patients[91].

Possible adverse effects of specific COX-2 inhibitors on bone metabolism can cause great socio-economic loss and interfere with standard treatment guidelines of chronic pain disease in the elderly. In addition, patients in older age groups are people who most need dental implant sfor their quality of life. This group not only has Rheumatoid arthritis or Osteoarthritis, but also metabolic bone disease of osteoporosis that can have adverse effects on bone healing and osteointegration of dental implants[84].

Dental implants provide means of anchoring various oral prostheses. The success of titanium and its alloy implants has resulted in their routine use in dentistry. A fundamental factor for a successful implant is good cancellous bone surrounded by cortical bone of adequate thickness around the implant. However, success of implants is multi-factorial and bone metabolism is one of many important factors[92]. Although, preliminary results of a retrospective study of over 200 patients over 65 years of age conducted at the Prosthodontics Intermedica indicated that age was not a factor influencing success of the dental implants[93]. Medications and systemic disease in this age group may contribute greater effects on bone healing and osteogenesis than in younger age groups.

Due to the wide prescription of celecoxib in treating bone related disorders, understanding the *in vitro* effect of celecoxib on osteoblast is crucial[94]. There are few publications that directly address effects of specific COX-2 inhibitors on osteointegration of dental implants. There is no study directly investigating effects of specific COX-2 inhibitors in therapeutic doses on growth and differentiation of osteoblasts on titanium surfaces.

Objective of the study

Aims of The study

1. To investigate the effects of a specific COX-2 inhibitor, celecoxib, on growth, differentiation and PGE_2 synthesis of osteoblasts cultivated on smooth surface (acid prickle) titanium disks;
2. To compare the effects of a specific COX-2 inhibitor, celecoxib, and a non specific COX-1 and COX-2 inhibitor, indomethacin, on function of osteoblasts cultivated on smooth surface (acid pricked) titanium disks
3. To study dosage and time dependent effects of celecoxib and the influence of stages of cell growth and the effects of celecoxib upon osteoblasts cultivated on smooth surface (acid pricked) titanium disks and

Hypothesis

It was hypothesized that (1) therapeutic doses of specific COX-2 inhibitors NSAIDs reduced prostaglandin synthesis and inhibited differentiation of osteoblasts in dose and time dependent manner; (2) The reduction of growth and differentiation of osteoblasts was directly associated with the reduction of prostaglandin synthesis and stages of cell growth; (3) Inhibitory effects of specific COX-2 inhibitors NSAIDs on osteoblasts were greater than effects of non-specific COX-2 inhibitors or conventional NSAIDs.