D-002 (Beeswax Alcohols): Concurrent Joint Health Benefits and Gastroprotection

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Molina *et al.*: D-002 on Joint Health and Gastroprotection

Nonsteroidal antiinflammatory drugs include the traditional drugs and more selective COX-2 inhibitors. Traditional nonsteroidal antiinflammatory drug use is hampered by their gastrotoxicity, while COX-2-inhibitors increase the cardiovascular risk. The search of safer substances for managing inflammatory conditions is updated, a challenge wherein dual COX/5-LOX inhibitors have a place. This review summarizes the benefits of D-002, a mixture of higher aliphatic beeswax alcohols, on joint health and gastric mucosa. D-002 elicits gastroprotection through a multiple mechanism that involves the increased secretion and improved quality of the gastric mucus, the reduction of hydroxyl radical, lipid peroxidation, protein oxidation, neutrophil infiltration and the increase of antioxidant enzymes on the gastric mucosa. Consistently, D-002 inhibits NSAIDs, ethanol, pylorus-ligation and acetic acid-induced gastric ulceration in rats, and has reduced gastrointestinal symptoms in clinical studies. Early results found that D-002 was effective in the cotton pellet-induced granuloma and carrageenan-induced pleurisy model in rats, lowering pleural leukotriene B4 levels without causing gastrointestinal ulceration. However, D-002 effects on inflammation received little attention for years. Recent data have shown that D-002 inhibited both COX and 5-LOX activities with a greater affinity for 5-LOX and could act as a dual COX/5-LOX inhibitor. This mechanism might explain efficacy in experimental inflammatory and osteoarthritic models as well as clinical efficacy in osteoarthritic patients while supporting the lack of D-002 gastrotoxicity, but not the gastroprotective effects, which appear to be due to multiple mechanisms. In summary oral D-002 intake could help manage inflammatory conditions that impair joint health, while offering gastroprotection.

Key words: Antiinflammatory agents, beeswax alcohols, D-002, gastroprotection, joint diseases, osteoarthritis

Inflammation is a host response from tissues that try to repair the damage elicited by noxious stimuli and to restore the normal functioning[1,2]. Acute inflammation, often accompanied by pain, provides the ability to return to normality after battling against internal or external foreign stimuli[2–4]. The processes of resolution and repair that happen during acute inflammation require activating endogenous signals that switch from producing proinflammatory towards proresolving molecules. If uncontrolled, acute inflammation may lead to pathological chronic inflammation linked with many diseases, including degenerative illnesses, autoimmune diseases and many malignancies[2-9]. Arachidonic acid released from membranes is metabolized by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 enzymes into eicosanoids. The action of COX, which leads to the production of prostaglandins (PGs) and thromboxane, involves two main isoforms: COX-1 and COX-2, which are mainly linked with physiological and pathological processes, respectively. In turn, LOX pathway action leads to the production of pro-inflammatory byproducts, like the leukotrienes (LT)[10].

Nowadays, nonsteroidal antiinflammatory drugs (NSAIDs) commonly used to treat inflammation worldwide, include the traditional NSAIDs (t-NSAIDs) that inhibit COX-1 and COX-2, but whose antiinflammatory and analgesic effects are mostly COX-2-dependent; and the selective COX-2 inhibitors. The use of t-NSAIDs, however, is relatively limited by their gastrotoxicity due to the inhibition of COX-1 in gastrointestinal cells that curtails the production of gastroprotective PGs and leads to the increased production of proinflammatory and gastrotoxic LT through the LOX pathway[10-13].

COX-2 selective inhibition, designed to minimize the gastrointestinal toxicity of t-NSAIDs, contributes to

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an imbalance between prostacyclin and thromboxane production in vascular cells, and to develop hypertension and atherosclerosis, and that increase the cardiovascular risk[14-18]. Randomized controlled trials have shown that the risk of t-NSAID-induced gastropathy can be reduced by the concomitant intake of proton-pump inhibitors (PPI) or misoprostol[19], but poor adherence to this scheme among users of t-NSAIDs or low dose aspirin is common[20].

Then, safer strategies have been explored to treat patients with chronic inflammatory diseases[21], including nonpharmacological therapy as the first option to manage osteoarthritis[22–24]. Since many chronic inflammatory diseases are health style-related, they could be prevented by the control of modifiable risk factors and adherence to healthy lifestyles[25,26]. In addition, the use of dual COX and 5-LOX inhibitors is another strategy to manage inflammation without producing gastrotoxicity[27]; which could be complemented by some promising products that have shown both antiinflammatory and gastroprotective effects in experimental studies, but evidence of their clinical efficacy is still lacking[28-31]. In light of these findings, search for new substances that might help manage inflammatory conditions safely is ongoing. This review attempts to summarize the evidences that support the benefits of D-002 (beeswax alcohols) on joint health and gastric mucosa.

EXPERIMENTAL GASTROPROTECTIVE AND ANTIOXIDANT EFFECTS

D-002 is a mixture of six high molecular weight alcohols purified from the beeswax (*Apis mellifera*, L) with the following composition, tetracosanol (6–15%), hexacosanol (7–20%), octacosanol (12–20%), triacontanol (25–35%), dotriacontanol (18–25%), tetratriacontanol (≤7.5%) (purity ≥85%)[32].

D-002 research started in the nineties, when experimental data documented that oral D-002 treatment prevented HCl-, ethanol-, NSAIDs-, aspirin-, water-stress restraint- and pylorus ligation-induced gastric ulceration in rats without modifying gastric acidity, indicating that it does not act by acid suppression[33,34].

Many studies were conducted thereafter to elucidate the mechanisms by which D-002 protected gastric mucosa against aggressive agents. Experimental studies have revealed that the gastroprotective effect of D-002 comprised of a multiple, rather than a single mechanism, which increased secretion and improved quality of the gastric mucus, based on the effects demonstrated in rats with and without ethanol ulcers[34,35]; antioxidant effects exerted on the gastric mucosa[36,37], and a reduction of neutrophil infiltration in the gastric mucosa in rats with NSAIDs-induced acute gastric ulceration[38].

The antioxidant effects of D-002 on the gastric mucosa provided a relevant mechanism of gastroprotection. Oral acute treatment with D-002 (5-100 mg/kg) has been shown to reduce gastric ulceration and malondialdehyde (MDA) formation, a marker of lipid peroxidation, in the gastric mucosa of rats with indomethacin[36,37] or ischemia reperfusion-induced ulcers[36]. Also, D-002 (5-100 mg/kg) lowered carboxyl groups (a marker of protein oxidation), the generation of hydroxyl (*OH) radical and myeloperoxidase (MPO) activity (a marker of inflammation), and increased catalase (CAT) activity in the gastric mucosa of rats with indomethacin ulcers, while only the highest dose (100 mg/kg) increased glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) activities[37].

These results are in line with the antioxidant effects of D-002 demonstrated experimentally in rat tissues plasma, liver and brain, wherein it was able to reduce lipid peroxidation and protein oxidation, and raised antioxidant enzyme activities[39,40]. Clinical studies have found that D-002 lowered lipid peroxidation and protein oxidation markers; and increases the plasma total antioxidant status (TAS, a marker of the body's antioxidant response)[41–44]. Besides, reduced neutrophil infiltration in the gastric mucosa and increased restorative angiogenesis to the ulcerated areas support the healing effect of D-002 on acetic acid chronic gastric ulcers in rats[45].

Further more, oral treatment with D-002 has also been shown to protect against cysteamine-induced duodenal ulcers in rats[46]. Also, a recent study revealed that in addition to its ability to prevent gastroduodenal ulceration induced by noxious stimuli, D-002 protected against reflux esophagitis and prevented the increase of esophageal concentrations of lipid peroxidation and protein oxidation markers in a model of acute gastroesophageal reflux induced in rats[47].

CLINICAL STUDIES

In parallel, various randomized, double-blinded, placebo-controlled clinical (RDBPC) studies were undertaken to investigate whether D-002 displayed gastroprotective effects in humans. Two of these studies demonstrated that D-002 (100 mg/day) given for short-term (8 weeks) improved gastrointestinal symptoms, assessed by the Gastrointestinal Symptom Rating Scale (GSRS), since it reduced the overall GSRS score, and acidity/heartburn, regurgitation, bloating, sucking and flatulence scores versus placebo[48,49]. Other trial found that D-002 (40 and 100 mg/day) given for 2 weeks decreased gastrointestinal adverse events and specifically acidity/heartburn in subjects with osteoarthritic symptoms treated with 20 mg/day of piroxicam[50]. Also, D-002 (100–150 mg/day) given for 6 months produced persistent gastroprotective and antioxidant effects in middle-aged and elderly subjects[51]. Moreover, an open study conducted on 184 middle-aged and elderly subjects treated with D-002 50 mg/day (168 cases), 100 mg/day (13), or higher doses (3) from 15 days to 6 years found that it improved heartburn and health perception[52].

BENEFICIAL EFFECTS ON JOINT HEALTH

An early paper also reported that single oral doses of D-002 were effective in acute and chronic inflammation models, like carrageenan-induced pleurisy (CAP) and cotton pellet-induced granuloma in rats, respectively, without causing gastrointestinal ulceration, and that it markedly reduced the concentrations of LTB4 in the pleural exudates of rats with CAP[53].

LTB4 is a powerful pro-inflammatory mediator that activates and recruits neutrophils to the site of injury, increases superoxide generation, pro-inflammatory cytokines production and may sustain bronchoconstrictor and gastrotoxic effects[54]. Such results then were promising and consistent with previous reports of the antiinflammatory effects of triacontanol, the main component of D-002, demonstrated by using *in vitro* and *in vivo* models[55,56].

Despite the attractive issue of having found a substance that reduced inflammation without producing gastrotoxicity, the effects of D-002 on inflammation received little attention for years. Years later, taking into account the spontaneous reports of joint pain relief in subjects consuming D-002 for managing their gastrointestinal symptoms[52], and the early demonstration of its antiinflammatory action[53], new studies have investigated the potential benefits of D-002 on joint health.

IN VITRO STUDIES

Recent data have shown that D-002 inhibited both COX and 5-LOX activities *in vitro*, with greater affinity for 5-LOX, so that it acts as a dual COX/5-LOX inhibitor[57,58]. This mechanism may explain, at least partially, the ability of D-002 for reducing experimental inflammation and osteoarthritis, and for alleviating osteoarthritis symptoms, and may support its lack of gastrotoxicity, but it alone cannot explain the gastroprotective effects D-002, which depend of the multiple mechanisms referred above: improved mucus secretion and quality, antioxidant effects and reduction of neutrophil recruitment on the gastric mucosa, increased restorative angiogenesis in ulceration sites[34–45].

IN VIVO EFFECTS ON EXPERIMENTAL INFLAMMATION AND JOINT FUNCTION

New studies have confirmed the antiinflammatory effects elicited by the oral acute administration of D-002 in models of acute inflammation[59–62]. Three hours after dextran administration paw edemas were reduced significantly by D-002 (200-800 mg/kg, up to 71.4%) and indomethacin 10 mg/kg, the reference treatment (52.3% inhibition). The effect of D-002 (800 mg/kg) was greater than that of indomethacin. Also, histamine-induced plantar edema was significantly inhibited by D-002 (200-800 mg/kg) to 57%, and a roughly similar reduction (60%) was produced by diphenhydramine 60 mg/kg, the reference substance. D-002 (200-800 mg/kg) moderately (36%) inhibited serotonin-induced plantar edema, which was markedly inhibited (79%) by cyproheptadine 10 mg/kg, the reference drug[59].

Oral treatment with D-002 has been shown to reduce dose-dependently (50-400 mg/kg) the edema and MPO activity in the model of xylene-induced edema in mice ear. Topical application of D-002 (2.5-10%),

however, was ineffective in this model, which leads to conclude that acute oral, but not topical administration of D-002, was effective to decrease xylene-induced mouse ear edema[60]. These studies support that single oral doses of D-002 were effective in experimental models of acute inflammation.

Other two studies expanded the knowledge of the effects of D-002 on the model of CAP in rats[61,62]. Earlier results had shown the efficacy of D-002 for lowering the edema and LTB4 pleural concentrations in rats with CAP, a model of acute inflammation characterised by lung neutrophil infiltration and increased lipid peroxidation, but its effects on these targets were not studied. A new study investigated the effects of D-002 on neutrophil infiltration and lipid peroxidation in the lung tissue in rats with CAP, and found that D-002 acute treatment (50, 200, 400 and 800 mg/kg) lowered practically abolishing neutrophil infiltration (27.4-99.9%) and MDA (72.5-96.3%) levels in lung tissues, and moderately reduced (≅31%) the volumes of pleural exudates versus the positive control. Aspirin 150 mg/kg reduced markedly neutrophil infiltration (90.2%) and moderately (46%) exudate volumes, but unchanged MDA values[61].

Neutrophils are major effectors of acute inflammation and also contribute to chronic inflammation and adaptive responses[63], so that these data reinforce the relevance of D-002 antiinflammatory effects. Since lyprinol, a lipid extract of the green-lipped mussel (*Perna canaliculus*), has been widely used to manage inflammatory joint conditions, another study compared the effects of acute oral administration of D-002 and lyprinol on edema formation, MPO activity and protein content in the pleural exudates of rats with CAP. Results showed that treatment with D-002 or lyprinol at doses of 50, 100 and 200 mg/kg, reduced significantly, dose dependently and moderately (≤30%) the volumes of pleural exudates as compared to the positive control. Both treatments lowered significantly MPO activity, but failed to modify the total protein content of the exudates. So, D-002 was as effective as lyprinol to reduce edema formation and MPO activity in the pleural exudates in rats with CAP[62].

Since acute inflammation commonly course with pain, and NSAIDs may ameliorate both inflammation and associated pain, other studies investigated if, in addition to its antiinflammatory effects on acute inflammation models, D-002 should exhibit analgesic effects[64,65]. One of these studies assessed the antiinflammatory effects of repeated (15 days) doses of D-002 on the xylene-induced mouse ear oedema, a model of acute inflammation, and explored its effects on models of analgesia and motor performance in mice. D-002 (25-200 mg/kg) reduced the edema (44.7-76.4%) and the increased MPO activity (38.0-57.0%) induced by the topical application of xylene on mouse ear, while indomethacin, the reference substance, inhibited edema and MPO activity by 59.9% and 57.5%, respectively. The highest edema reduction (76.4%) with D-002 was greater than the highest inhibition of MPO activity (57.0%), which suggests that mechanisms others than MPO inhibition, and then than neutrophil infiltration, are involved in its antiedema action[64].

The same study investigated whether acute repeat (15 days) doses of D-002 produced analgesic effects and affected mice motor performance. Analgesic effects were assessed with two methods: the acetic acid-induced writhing response (AAWR) and the hot plate response. The AAWR, a model of visceral pain used for evaluation of peripheral analgesics, involves the release of PGs and mediators like PGE2 and PGE2 that become increased in peritoneal fluid, a mechanism obviously linked to inflammation. In contrast, the hot plate is a model of neuropathic pain induced by acute thermal nociception specific for central antinociceptive drugs. Effects on motor and coordination performance were evaluated in the open field and rotarod tests. Single and repeat doses of D-002 (25, 50 and 200 mg/kg) decreased the AAWR (21.2, 28.2 and 40.1%, respectively) (*single doses*); (25.2, 35.1 and 43.2%, respectively) (*repeat doses*), without affect the hot plate, open field and rotarod behaviors. These data indicate that the analgesic activity of D-002 seen in the AAWR should be related to its anti-inflammatory effect[64].

Other study compared the analgesic effects of single oral doses of D-002 (25, 50, 200 and 400 mg/kg), naproxen (10, 20 and 50 mg/kg), aspirin (50,100 and 300 mg/kg) and paracetamol (100 and 400 mg/kg) in mice. AAWR were significantly lowered by D-002 (200 and 400 mg/kg) (43 and 40%, respectively); aspirin 300 mg/kg (83%); naproxen 50 mg/kg (37%) and paracetamol 400 mg/kg (24%).

The lowest dose of each treatment was ineffective, aspirin 300 mg/kg the most effective, D-002

400 mg/kg and naproxen 50 mg/kg similarly effective and paracetamol 400 mg/kg was the less efficacious treatment[65].

Current management of chronic inflammation often includes NSAIDs therapy. Since D-002 ameliorates NSAIDs-induced gastrotoxicity, other study compared the effects of D-002, naproxen, aspirin and their combined therapy on the cotton pellet granuloma, a model of chronic inflammation, and the gastrotoxicity of each treatment, as well[66].

The study demonstrated that oral treatment with D-002 (50 and 200 mg/kg), naproxen (10 and 25 mg/kg) and aspirin (150 and 300 mg/kg) lowered significantly and similarly granuloma weights in rats (≅40% as compared to the positive control group). Naproxen and aspirin, but not D-002, induced gastric ulceration in the rats. The combination therapies D-002 50 mg/kg+naproxen 10 mg/kg or D-002 50 mg/kg+aspirin 150 mg/kg did not cause greater antinflammatory effects than each monotherapy, but the ulcerative effects of naproxen 10 mg/kg and aspirin 150 mg/kg were reduced by about 69% when D-002 50 mg/kg was given together with them, which suggests that it reduced NSAIDs gastrotoxicity[66]. Other two studies investigated the effects of oral treatment with D-002 on formaldehyde-induced and monosodium iodoacetate (MIA)-induced osteoarthritis in rats[67,68].

The effects of D-002 on the formaldehyde-induced osteoarthritis in rats were assessed by measuring the changes on the diameters of rat ankle and paw after 10 days on treatment. The formaldehyde injection increased these diameters in the positive controls as compared to the negative ones. Although all doses of D-002 (50-400 mg/kg) reduced the increases of rat paw diameters (26.9-30.8%), the effect was not dose-dependent, and somewhat lower than that of naproxen 3 mg/kg (46.1% inhibition). D-002 and naproxen were more effective for reducing ankle edema than paw edema. The maximal reduction of ankle edema with D-002 (51.7%), reached with 200 mg/kg, was lower than with naproxen (89.7%)[67].

The effects of oral administration D-002 for 10 days on MIA-induced joint damage were assessed by histology. MIA injection increased the depth, extent and histological scores of cartilage damage, changes that were ameliorated by D-002 (50, 200 and 400 mg/kg), which reduced the loss of chondrocytes and proteoglycans, pannus formation and joint inflammation. Ibuprofen 30 μmol/kg reduced joint inflammation and the depth of cartilage damage, not the other parameters. These results indicate a protective effect of D-002 against joint damage in MIA-induced osteoarthritis in rats[68].

CLINICAL STUDIES

In light of the experimental data, a RDBPC study investigated the effects of D-002 (50 mg/day) on patients with osteoarthritis symptoms randomized to D-002 (50 mg) or placebo for 8 weeks. The primary efficacy outcome was the reduction of the total Western Ontario and McMaster Individual Osteoarthritis Index (WOMAC) score. The decrease on pain, joint stiffness, physical activity scores, and use of rescue medications were secondary outcomes. D-002 significantly reduced the total WOMAC score (38.0%) and the pain (30.6%), joint stiffness (26.5%) and physical activity (41.2%) sub-scores versus placebo. Benefits were seen as soon as after 2 weeks on therapy. The use of rescue medication (paracetamol, metamizole) in D-002 group (3/30, 10%) was lower than in placebo (10/30; 33.3%).

Treatment was well tolerated. Four subjects (3 D-002-treated, 1 placebo) discontinued the study, none due to adverse experiences (AE), while 5 (2 D-002, 3 placebo) reported mild AE. This study concluded that D-002 (50 mg/day) given for 8 weeks improved osteoarthritis symptoms, being well tolerated[69].

Another RDBPC study investigated the effects of D-002 (50 to 100 mg/day) given for just 6 weeks to subjects with osteoarthritis symptoms randomized to D-002 (50 mg) or placebo. Symptoms were assessed by the WOMAC and the visual analogy scale (VAS) scores. Subjects without symptom improvement at week 3 were titrated to two daily tablets. The primary outcome was the decrease on the total WOMAC score. WOMAC pain, joint stiffness and physical function scores, VAS score and use of rescue medications were secondary outcomes. Twenty-three subjects (2/30 D-002, 21/30 placebo) needed dose titration. At trial completion D-002 lowered total (65.4%), pain (54.9%), joint stiffness (76.8%) and physical function (66.9%) WOMAC scores, and

VAS score (46.8%) versus placebo. These decreases, significant from the 2nd week, were enhanced over the trial. Less D-002-treated (6/30) than placebo subjects (17/30) required rescue medications. There were not study withdrawals and the treatment was well tolerated. Summarizing, D-002 (50–100 mg/day) given for 6 weeks ameliorated arthritic symptoms and was well tolerated[70].

SAFETY, EXPERIMENTAL TOXICOLOGY

Toxicological studies conducted in rodents and non-rodents support that D-002 is safe, and failed to find D-002-related toxicity[71–78]. The highest dose (1000 mg/kg) in the chronic study in rats, which did not cause related toxicity, was 1408 and 345 times greater than the minimal (50 mg/day, ≅0.7 mg/kg for an average body weight of 70 kg) and maximal (200 mg/day, ≅2.8 mg/kg) human doses. Experimental toxicology evaluations demonstrated that D-002 is safe even at 1000 mg/kg (≅1408 times greater than the standard human dose).

CLINICAL SAFETY

Safety and tolerability analyses have included withdrawal analysis, effects on safety indicators and AE reports. Physical examinations have been performed at each visit and laboratory tests at baseline and study completion. Specific algorithms to classify AE as mild, moderate or severe were used. Most studies have been RDBPC[39-44,48-51,69,70], but safety data of an open study agree with the tolerability seen in controlled studies[51].

Clinical safety studies of D-002 (50-200 mg/day) supported that it did not affect physical or blood safety indicators, and did not induce AE different from those occurred in placebo treated subjects. Data of AE reported during post-marketing surveillance (PMS) studies were consistent with the good tolerability profile of D-002 shown in clinical trials.

CONCLUSIONS

Overall, experimental studies have shown the ability of D-002 for lowering inflammation through mechanisms that include the inhibition of COX and 5-LOX enzymes, the reduction of the harmful LTB4 and antioxidant effects. Also, D-002 has been shown to ameliorate experimental osteoarthrtitis, improving all aspects of joint damage (cartilage degeneration, pannus formation, joint inflammation). Consistently, RDBPC studies demonstrated that D-002 help relieve osteoarthritis symptoms. On the other hand, D-002 may provide gastroprotection by reinforcing defensive factor of the gastric mucosa, like improved quality and increased mucus secretion, and a set of antioxidant effects at such level. RDBPC studies have demonstrated that D-002 reduced gastrointestinal symptoms in middle-aged and older subjects. Experimental and clinical data indicted that D-002 could be safe. These properties make D-002 a good candidate to help manage inflammatory conditions without the risk of gastrotoxicity, or even offering gastroprotection.

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