

Anti-inflammatory Activity of an Oil Derived from *Perna canaliculus* (New Zealand Green-lipped Mussel).

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Citation

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Abstract

Background: There is considerable evidence that the New Zealand green-lipped mussel has beneficial effects for the treatment of chronic joint conditions through its anti-inflammatory activity. A new oil product based on an extract from the mussel has been prepared and its efficacy and possible mechanism of action for alleviating joint conditions investigated. **Method:** The effect of the oil on the production of superoxide by activating primary rat neutrophils in culture was measured. The dose-response effectiveness of it in reducing the inflammatory response in pristane-induced rheumatoid arthritis in rats was assessed. The results were statistically significant in both cases. **Results:** The oil was shown to suppress superoxide production by activated neutrophils by 32% even when diluted 4000 fold. When administered orally to rats it significantly inhibited the development of rheumatoid arthritis even at doses as low as 125 mg per kg of food. **Conclusions:** This oil has a potent effect on the production of superoxide by activated neutrophils and also is a strong inhibitor of rheumatoid arthritis in rats, probably through its anti-inflammatory activity.

BACKGROUND

Arthritis is a term that refers to a number of conditions that involve damage and destruction of joint tissue. Two of the most common types are osteoarthritis and rheumatoid arthritis. The former involves an abnormal wearing of the cartilage that provides the protection for the bones. As this wear progresses the pain and inflammation associated with movement and weight bearing increases. The latter arises from an auto-immune reaction that results in inflammation and destruction of joint tissue.

A number of cellular and molecular factors have been identified that are key to the progression of arthritis. Neutrophils, monocytes and macrophages have all been implicated. Each of them produces a range of pro-inflammatory mediators which include the tumour necrosis factor- α (TNF- α), reactive oxygen species such as superoxide and the COX-2 pathway.

Although these are major chronic health conditions that afflict a significant proportion of the population, the incidence of both is increasing. Much of the suffering associated with it is the pain and accompanying inflammation. The identification and characterisation of agents that can alleviate this are areas of active investigation [1 – 3]. There are a number of drugs (pharmaceuticals)

which have this aim, with many of them directed towards inhibiting COX enzymes [4, 5] or inhibiting the action of inflammatory mediators such as TNF- α [1, 6 – 8]. The COX-2 isozyme is inducible in a diverse range of inflammatory conditions including arthritis. A number of pro-inflammatory mediators have been identified in arthritic joints with TNF- α concentrations being significantly elevated.

Another class of active agents is based on natural products. There are a number of these with an established reputation [9 – 11]. Among them are products based on the New Zealand green-lipped mussel (*Perna canaliculus*). It has a well-documented reputation for treating inflammation [12 – 17]. It has been shown in both experimental animal studies and human trials to be beneficial for the treatment of arthritic conditions [18 – 23]. Despite extensive research the active constituent(s) has not been identified. The anti-inflammatory effect appears to be quite potent compared with other species of mussel. While the active constituents have not been positively identified, there are numerous derivatives and fractions of the original preparation that are being marketed. These are claimed to have improved properties such as greater efficacy, palatability or reduced toxicity. One such preparation has been used in these experiments. It is a mixture of Pernoil™ (a proprietary mussel oil extract) and

olive oil (1:2, v:v) and is designated as P/O. P/O is a strong antagonist of the inflammatory activity of activated neutrophil cells and when administered orally is a very potent inhibitor of the inflammatory response in a rat model of rheumatoid arthritis.

MATERIALS AND METHODS

NEUTROPHIL ISOLATION

The assay of the effect on inflammation in activated neutrophils is based on the method of Tan and Berridge (2000) [24]. Neutrophils were prepared from freshly isolated rat blood by collecting in EDTA tubes. The blood was layered over an equal volume of Polymorphoprep (Axis-Shield PoC AS, N-0504 Oslo, Norway) and centrifuged at 500g for 30 min at 20°C. The polymorphonuclear cells were isolated and suspended in Hanks Balanced Salt Solution (HBSS) and centrifuged at 350g for 5 min at 4°C.

The cell pellet was washed with HBSS and then resuspended in HBSS (10⁶ cells per ml). 140 µl of the cell suspension was added to individual wells of a 96 well microplate while blank wells contained 140 µl of HBSS. Triplicate 20 µl aliquots of the test solutions were added to the wells containing cells and to blank wells. Each test solution was assayed at three concentrations.

SUPEROXIDE ASSAY

A triplicate set of wells containing 20 µl of HBSS instead of sample served as the controls and the blanks. As well, acetylsalicylic acid (Sigma, St Louis, USA) was included as a positive control.

20 µl of PreMix WST-1 (Takara Bio Inc, Otsu, Shiga, Japan) was added to each well followed by 10 µl of catalase (1mg/ml, Sigma). Following 15 min pre-incubation at 37°C, the cells were activated by adding phorbol myristate acetate (Sigma, final concentration 50 µg/ml). The microplate was incubated at 37°C in 95% air/5%CO₂ for 2 hr. The absorbance in each well was measured at 450nm and the degree of inhibition or stimulation of superoxide produced resulting from the addition of the test products was calculated.

IN VIVO STUDIES

The methodology is based on the development of rheumatoid arthritis in rats following the injection of pristane (Sigma, St Louis, USA) [25, 26]. Each group of female Dark Agouti rats (17.5 to 18.3 weeks of age) comprised six animals. There were three experimental

groups which had P/O added to their diet by being incorporated in their food pellets. While groups A and E received unsupplemented food, group B had P/O at 125mg per kg of food, group C had P/O at 250mg per kg of food and group D was fed food supplemented at 375mg per kg. Food was available ad lib to all rats for the duration of the study. The rats were housed in groups of two.

After three weeks of feeding, arthritis was induced by the injection of Pristane intradermally (3 injections, each of 50 µl of pristane) at the base of the tail of each rat. At the same time and every day thereafter, Meloxicam (Boehringer Ingelheim, Mannheim, Germany) was administered by oral gavage to each rat of group E at a dose of 0.12 mg per kg body weight.

Body weights were recorded regularly and from the tenth day after the pristane administration, daily measurements of the volume displacement of each hind foot were taken by plethysmometry and the swelling calculated as a percentage change compared with the values obtained at the time of pristane administration. As well the inflammation in all four feet was scored daily according to the procedure described by Larsson et al. [27] and Kawahito et al. [28]. Each joint was scored on a scale of 0-3 by an observer for the degree of oedema and of erythema. This protocol was approved by the Animal Ethics Committee of the University of Otago, Wellington, New Zealand.

STATISTICAL ANALYSES

The data were analysed by either Student t-test for in vitro studies or by bivariate ANOVA (in vivo studies) (p<0.05 being significant).

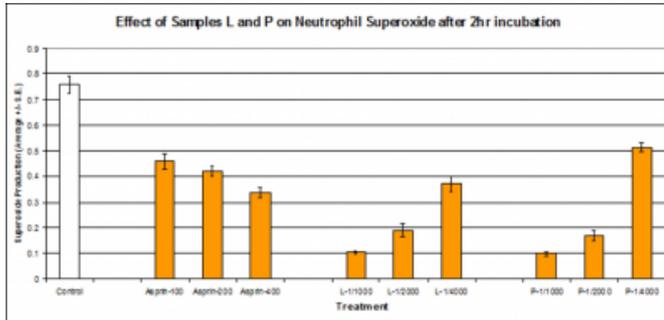
RESULTS AND DISCUSSION

Activated Neutrophil Experiment

The effects of the P/O, the Lyprinol and the acetylsalicylic acid on the production of superoxide by activated neutrophils are summarised in Figure 1. Each was assayed at three concentrations.

Figure 1

Superoxide production by activated neutrophils. Superoxide production by neutrophils exposed to several concentrations of Lyprinol, P/O and acetylsalicylic acid is expressed as a percentage of the control (untreated) cells.

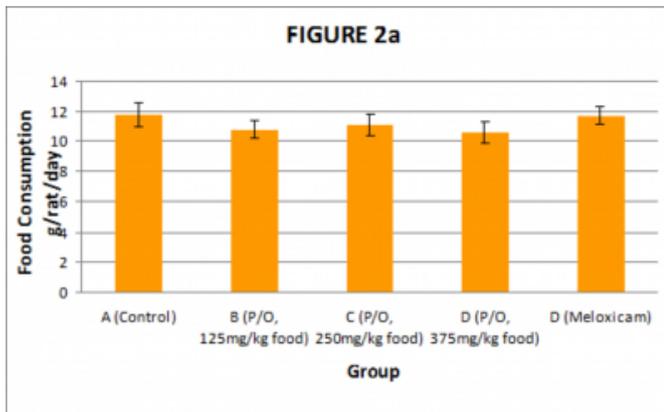


All three showed a dose response pattern of inhibition. Compared with the control incubations all inhibitions were statistically significant.

ANIMAL STUDIES

The food consumption was virtually identical for all groups (Figure 2a).

Figure 2

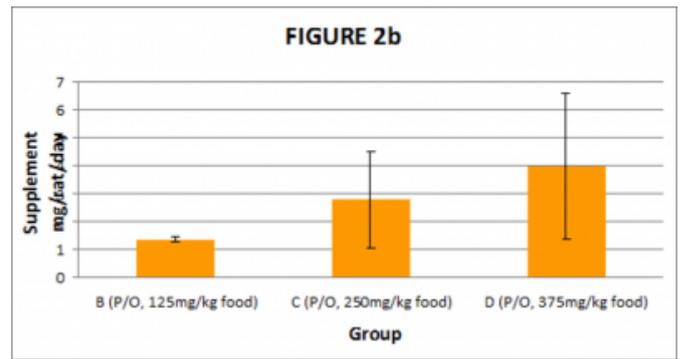


Mean daily food consumption by rats. Expressed as mean ± SEM with n=6 for each group.

The average daily consumption of P/O by each of the groups was calculated from this

(Figure 2b).

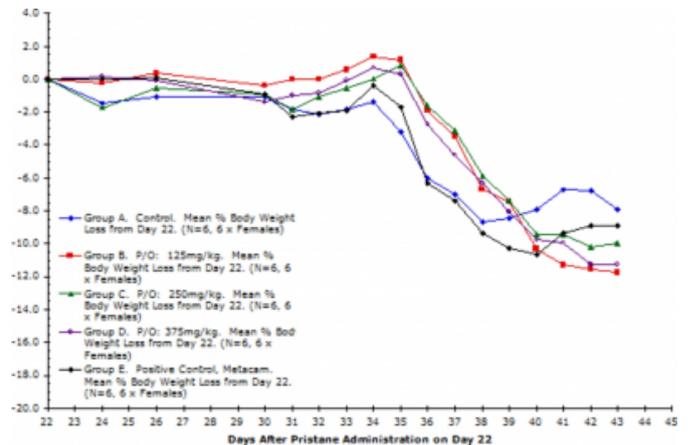
Figure 3



Mean daily supplement consumption by rats. Expressed as mean ± SEM with n=6 for each group.

The changes in body weight of the rats of the various groups are summarised in Figure 3.

Figure 4

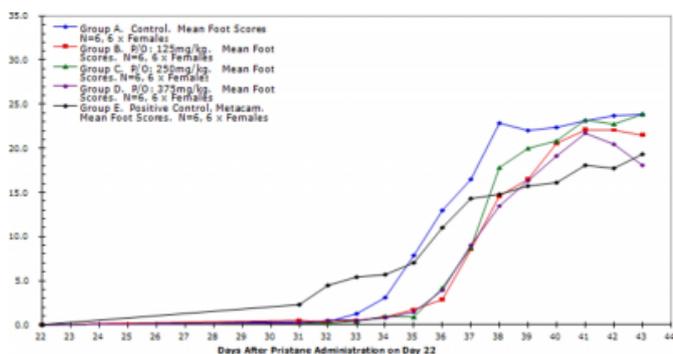


Body weight losses. Percentage of original on Day of Pristane administration. Comparison of Groups

A (Control), Group B (P/O: 125mg/kg), Group C (P/O: 250mg/kg), Group D (P/O: 375mg/kg) & Group E (Metacam).

The degree of inflammation of all joints nominated was measured for each rat at regular intervals and a score was assigned at each time point as described in Methods above. These are summarised in Figure 4.

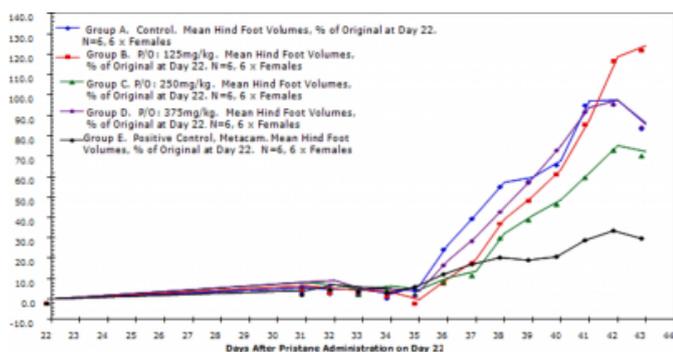
Figure 5



Foot scores following Pristane administration. Comparison of Group A (Control) vs Group B (P/O: 125mg/kg), Group C (P/O: 250mg/kg), Group D (P/O: 375mg/kg) & Group E (Metacam).

The changes in foot volumes were measured regularly after the injection of the pristane. For each of the five groups these changes are summarised in Figure 5.

Figure 6



Hind foot volume changes following Pristane administration. Comparison of Group A (Control) vs Group B (P/O: 125mg/kg), Group C (P/O: 250mg/kg), Group D (P/O: 375mg/kg) & Group E (Metacam).

Joint inflammatory conditions are a major burden on health systems in many countries. A number of these are chronic and become more significant as the sufferers age. As the longevity of humans is universally increasing, treatment of these conditions is assuming increasing importance.

There are a number of pharmacological treatments being developed and offered. Some of these are based on single molecule species; others are based on the modulation of inflammatory reactions.

There is a strong interest in the use of natural products or preparations derived from natural resources for treating joint conditions. These include chondroitin and glucosamine [9],

cat's claw (*Uncaria tomentosa*) [10], omega-3 fatty acids [29] and evening primrose oil [30]. It has been known for a number of years that the New Zealand green-lipped mussel (*Perna canaliculus*) is a source of such activity. This has been shown repeatedly [12 – 16]. Despite much effort, no active molecular species has been identified. The activity has been ascribed to multiple constituents acting in concert and the nature of these has not been fully characterised. Pernoil is an extract that is derived from *Perna canaliculus*.

In arthritic conditions, the neutrophil cell is well characterised as one of the major inflammatory cells that has been recruited and accumulated in the joint [31]. It becomes activated and, as a consequence, releases a number of pro-inflammatory compounds including tumour necrosis factor- α (TNF- α), nitric oxide (NO) and reactive oxygen species [32]. Inhibition of this activation is likely to have an anti-inflammatory effect. In the study reported here, P/O clearly displayed a dose response inhibition of the production of superoxide. A 1:1000 dilution of the oil reduced the production by 87% – a highly potent effect. Even at 1:2000 this antagonism was still nearly 78%. These effects were essentially identical to the potencies of another green-lipped mussel based product, Lyprinol. Even when diluted 4000 fold, P/O was still able to inhibit the production by 32%. All of these effects were highly significant statistically. By way of comparison the well-known anti-inflammatory compound, acetylsalicylic acid, at 100 μ g/ml produced 39% inhibition.

This oil (P/O) was administered orally to rats that had been injected with pristane to induce a rheumatoid arthritis. It was incorporated into the food pellets at three different dose levels. This supplementation had little effect on the food intake and so it would seem that the animals accepted the oil as palatable and digestible. Consequently the administration of the P/O followed a dose response.

It is a characteristic of chronic joint inflammation models that there is a steady loss of body weight and general condition from about 12 days after the initiation of the inflammation [33]. However the body weights of the rats in the three experimental groups were maintained during the three weeks of feeding the supplements prior to the initiation of the joint disease. In fact, they lost less than the animals on the control unsupplemented diet. That is, it was apparent there were no adverse effects from the addition of the P/O to the diet. In fact it may be somewhat beneficial. There was no evidence of any toxicity or safety issues associated with the administration of the P/O. The weights of the rats in the three experimental groups in this study were slightly higher

than those of the control animals on day 22 and so as the joint condition developed, their weights were generally higher up to about Day 39 (Figure 3). Thereafter the control group weights stabilised while there was still a continuing loss for the other groups, although near the end of the study they also showed signs of levelling off. The reason for this is uncertain although it is noted that this loss is only with the groups supplemented with P/O.

The swelling of the joints as measured by the changes in the foot volumes showed that the lowest dose of P/O had some effect (Group B). There was a distinct inhibition for this group up to Day 40 (Figure 4) but beyond that there was no protection. So even at an average daily dose of 1.35mg, rats were protected, particularly in the early stages of disease development. When the dose was doubled, there was a significant reduction in the joint swelling (Group C) (Figure 4). For example at Day 37 the volume of the feet in these rats was approximately 67% less than for the untreated animals. The highest dose of the P/O (375mg per kg of food) was somewhat less effective than the 250mg per kg of food (Group C). This indicates that there was not a direct dose response over the concentration range tested. Rather this product may exhibit the phenomenon of hormesis where there is a defined concentration range where inhibitory activity is optimal (34-37). The known anti-inflammatory (anti-arthritis) drug, Meloxicam, was shown to be a good inhibitor which had a virtually identical inhibitory effect in this investigation.

The foot score measurements are an average of the effect of the test substance on the swelling and degree of inflammation of a multiple number of joints in all four feet. By this measure, all of the doses of P/O inhibited in a very similar manner as shown by the scores. The rats treated with the three doses of P/O showed identical scores up to Day 37. Up to this time point these scores were considerably less than for the untreated rats. On Day 35, for example, the scores were approximately 82% less than for the untreated group. Even on Day 37 they were about 47% less. Thereafter the lowest dose group (Group B) maintained a reduced score. For example on Day 38 it was 36% less. But by Day 40 the difference was only 8% and this score was maintained to the end of the study. So it appears that, based on the scoring system, the low dose of P/O was very strongly effective until Day 37 but after that the effect was considerably reduced.

Based on the scoring system, the 250mg of P/O per kg was less effective than the lower dose. On Day 38 it was 22%

less and this difference was reduced to only 7% on Day 40 and for the final few days of the study this concentration had no effect on the score.

The high dose of P/O (375mg per kg) was approximately as effective as the low dose up to Day 41. So it did have some effect on the scores. However, on the last two days of the study the score for this group steadily decreased. Thus there is an indication that there was not a direct correlation between the foot scores and the dose level. Hormesis may also be relevant for this measure also.

The Meloxicam had very little effect on the score up to Day 37 – the decrease was only 13% by this time. Thereafter it was more noticeably effective as anticipated. The rate of increase in the score was considerably less. On Day 38, for example, the score for this group was 35% less than for the control group, on Day 40 it was still 28% less, and on Day 43 it was 20% lower.

Following from the neutrophil studies that demonstrated a significant anti-inflammatory effect for P/O, the in vivo studies have shown that P/O is effective at alleviating the inflammatory response in a rat model of rheumatoid arthritis. The effective dose of 2.78 mg per rat per day translates to about 13 mg per kg body weight per day which would be an acceptable dose if this translated to human efficacy.

CONCLUSION

P/O inhibits the production of superoxide by activated neutrophil cells. Thus it appears to have significant anti-inflammatory activity. In a rat model P/O is a strong inhibitor of the inflammatory response associated with experimental rheumatoid arthritis, as its performance measured against the well-known drug, Meloxicam, demonstrated.

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