In vitro effects of a Green-lipped mussel preparation on inflammatory mediator production by equine peripheral blood mononuclear cells

Master thesis
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Abstract

Introduction and literature review There is a wide range of green-lipped mussel (GLM) formulations available on the market, aimed at both human and veterinary patients. These extracts are mostly targeted at patients suffering from osteoarthritis. The efficacy of these different formulations has been extensively studied *in vitro* and *in vivo* (in humans, rats, mice, and dogs). So far, research results were inconsistent. In some studies, beneficial effects were observed, although in others the effect of GLM was not significant. Formulations specifically for the equine patient are also available, but research on their efficacy in horses is lacking. Therefore, this study was done to examine the possible anti-inflammatory properties of GLM in an equine model. Specifically, the effectiveness of GLMax® was studied, a commercially available GLM extract. Using LPS-stimulated and unstimulated equine peripheral blood mononuclear cells (PBMCs), the influence of GLMax® on the production of inflammatory mediators was measured. The NSAID meloxicam, which is frequently used to treat osteoarthritis pain in horses, was included in the experiment as a positive control. The solvent of GLMax® was included as a negative control.

Materials and methods PBMCs were isolated from blood samples from 4 horses. Isolated PBMCs were used in three cell culture experiments and a cell viability assay. PBMCs were incubated and/or pre-incubated with three concentrations of either GLMax®, meloxicam, or the solvent of GLMax®. The cells were challenged with LPS to stimulate TNF-α and PGE_2 production. A cytotoxicity assay was also performed for all solutions to evaluate the effects on cell viability.

Results No consistent anti-inflammatory effects of GLMax® were demonstrated in this study. Results from different experiments were variable. There are indications that high concentrations of GLMax® reduces TNF-α production in the presence of LPS, but this result could not be repeated in the last experiment. In the absence of LPS, GLMax® generally increased TNF-α levels. Results for meloxicam and the solvent solution were irregular. PGE_2 levels increased in the presence of high concentrations of GLMax® and LPS. However, the positive control Meloxicam only decreased PGE_2 in two experiments. The solvent solution had no significant effect on PGE_2 production.

Conclusion In this study, it can be concluded that GLMax® shows some *in vitro* anti-inflammatory effect, at least in the case of TNF-α production in the presence of LPS, but TNF-α levels are increased by GLMax® when LPS is absent. PGE_2 levels increase in the presence of GLMax® and LPS, but not when only LPS is added and the positive control was not always able to decrease PGE_2 levels. There are indications that GLMax® contains components that stimulate inflammatory-mediator production *in vitro*.

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List of abbreviations

Abbreviation	*Definition*
5-HETE	5-Hydroxyeicosatetraenoic acid
AA	Arachidonic acid
COX	Cyclo-oxygenase
DHA	Docosahexaenoic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Eicosapentaenoic acid
FFA	Free fatty acid
GAG	Glycosaminoglycan
GLM	Green Lipped Mussel
HPLC	High-Performance Liquid Chromatography
IgG	Immunoglobulin G
IL	Interleukin
INF-γ	Interferon γ
IS	Internal Standard
LPS	Lipopolysaccharide
LOX	Lipoxygenase
LTB4	Leukotriene B4
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
PBMC	Peripheral Blood Mononuclear Cell
PGE_2	Prostaglandin E2
PL	Phospholipids
PMA	phorbol 12-myristate 13-acetate
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid arthritis
SE	Sterol esters
SFE	Supercritical fluid extraction
ST	Sterol
TG	Triglyceride
TNF-α	Tumor necrosis factor α
OA	Osteoarthritis

1. Introduction

In the early seventies, it was discovered that indigenous Maoris living on the New Zealand coast suffered less from arthritis than Maoris living in the inlands. This difference was probably due to the green-lipped mussel, *Perna canaliculus,* in the diet from Maoris living on the coast.[1,2] Since then, many commercial green-lipped mussel (GLM) extracts have become available. The first commercial GLM extract, Seatone®, was introduced in 1976.[2] However, initial research with this extract in the 70s and 80s produced poor results. The mussel extract used in these studies showed only moderate anti-inflammatory activity in rats, sometimes no activity was found at all.[1,4,9] Researchers discovered that heating during the GLM extract production process caused cell damage and activated enzymes, leading to the degradation of active components in the mussel extract.[1] A new extraction process was developed, in which heating of the mussel was avoided[1] and tartaric acid was added to stabilize the mussel components[9], resulting in the conservation of bioactive components. This stabilized GLM extract and many others have become available since then, for both human and veterinary patients.

Extensive research on GLM has been done after the development of the first extract, both *in vitro*[5,6,9-12] and *in vivo* in laboratory animals[1,4,5,13-17], veterinary patients[18-25], and humans[3,26,27]. Both *in vivo* studies with rats[13] and several *in vitro* models[5,9,10] showed that GLM extracts have anti-inflammatory properties. In a study with human patients suffering from osteoarthritis (OA), GLM had an overall positive effect on arthritic scores[28]. However, GLM studies produced moderate results in patients suffering from rheumatoid arthritis (RA)[27,28] and asthma [26]. In veterinary medicine, GLM has been studied in dogs suffering from OA[19,29], generally producing positive results. Results from these studies will be discussed in more detail later.

Although the potency of GLM was extensively studied, studies on its effectivity in horses are lacking; there is only one published study regarding GLM in horses with lameness due to OA[24]. The *in vitro* anti-inflammatory effects of GLM in equine cells has, to our knowledge, not been studied. Therefore, this *in vitro* study will investigate the effect of GLM on the inflammatory response in equine peripheral blood mononuclear cells (PBMCs) incubated with and without lipopolysaccharide (LPS). Moreover, these effects will be compared to the anti-inflammatory effects of the well-established non-steroidal anti-inflammatory drug (NSAID) meloxicam. As a preparation for the study, previous research on GLM has been reviewed to compare different mussel extracts and obtain a general idea of previous results.

2. Literature study

First, the GLM production processes and the resulting different extract types will be discussed. This will be followed by a description of the biochemical and bioactive components in GLM extracts. The different types of GLM extracts have been extensively studied *in vivo* and *in vitro*. Results from these previous studies will be discussed in the final paragraphs of the literature review.

2.1 GLM extracts

GLM extracts are produced in New Zealand, the only country where the green-lipped mussel is endemic.[28] After the mussel is harvested, anti-oxidants are added to stabilize mussel components, after which it is (often) processed through freeze-drying. Freeze-dried mussels are manufactured into a fine powder, and available as such or processed further to produce a lipid extract.[28] According to Coulson et al[28], the amount of several bioactive substances may vary depending on the life cycle and the diet of the mussel, and the harvest season.[28] However, in 2003 a study on GLM components was performed and it was found that there were no major differences between the composition of GLM extracts originating from three sites.[30] The only major difference in extract composition is between frozen mussel extracts and freeze-dried mussel extracts, but this was probably due to the absence of water after the freeze-drying process.[30] Bioactivity was not reviewed in this study, so differences in the concentration of bioactive substances remain unknown.[30]

At the moment there are several commercial nutraceuticals available, such as Perna®[5] and Biolane®[24], containing the freeze-dried whole mussel. A short overview of commercially available GLM extracts is given in table 1. There is also a fluid, stabilized whole GLM product which is not frozen or powdered, GLMax®, produced by Synofit Europe b.v. The exact production process for this fluid GLM extract remains undisclosed. Apart from whole mussel products, lipid-rich extracts from GLM are also available. As mentioned in the introduction, in 1976 the first GLM lipid extract, Seatone®, was produced but was ineffective.[2] This has led to the development of Lyprinol®, another lipid extract, produced by supercritical fluid extraction (SFE) from stabilized, freeze-dried mussel powder.[2,13] This extract also contains olive oil and vitamin E, which is added after extraction.[28] Since there is such a variation in extracts, and exact production processes are often unknown, there are many different doses advised by manufacturers. The different dose ranges will be discussed below.

2.1.1 GLM dose range

The dose ranges of GLM applied in published studies, often based on the manufacturer's advice, differ extremely. For lipid extracts, Coulson *et al*[28] report large differences between dosing in rat studies, (20 mg/kg bw/day up to 100 mg/kg bw/day) and dosing in human clinical trials (200-1200 mg/day). Estimating human bodyweight at 70 kg, this comes down to a dose ranging from 3 to 17 mg/kg bw/day, much lower than the rat dosages.[28] It is unknown how the lower human dose was established, but it has been suggested this choice could have been made based on differences in metabolism between humans and rats.[28] This, however, is no explanation for the dosing variety between other species.

The variation in dosing is also evident for the different whole mussel extracts. The manufacturer's advice for Biolane® is 1500 mg/day for humans; this comes down to a dose of 21 mg/kg bw/day for a person of 70 kg.[28] In another study, the manufacturer's recommended dose was reported to be 65 mg/kg bw/day for a whole mussel powder.[11] In a previous *in vivo* study with horses, whole mussel powder (Biolane®) was studied at a dose of 25 mg/kg bw/day.[24] The dosing advice for Equisin (which is a whole mussel liquid) from Synopet is 15 ml/day for a horse, corresponding with 525 mg GLM. For a 500 kg horse, this would be a dose of 1,05 mg/kg bw/day. This dose is also much lower than the dosing used in rat studies (i.e. Perna® was studied at 100mg/kg bw/day[5]) and the dosing mentioned above. It is unclear how these differences between manufacturer's advice, clinical trials, and animal studies came to be, but it could be due to variation in concentrations of bioactive components. It can also be partially explained by differences in extract types (whole mussel or lipid-rich extracts).[28]

Type	Brand (manufacturer)	Ingredients	Studied in
Whole mussel extracts	GLMax® (Synofit Europe b.v.)	Whole mussel product	This study (a study with Synofit®, which contains GLMax® amongst other ingredients, has been done[31])
	Perna® (provided by FoodScience Corporation, Essex Junction, VT, USA)	Lyophilized GLM powder	Lawson *et al*[5] Mani *et al*[10]
	Biolane®	Lyophilized pure GLM extract	Cazyer *et al*[24]
	Seatone®	Tartaric-acid stabilized, dried GLM flesh	Whitehouse *et al*[9]
Lipid-rich extracts	Lyprinol® (Pharmalink International Ltd., Burleigh Heads, QLD, Australia)	Lipid-rich, supercritical fluid carbon dioxide extract of the freeze-dried tartaric-acid stabilized GLM powder, olive oil, vitamin E[32]	Whitehouse *et al*[9,14], Lee *et al*[13], Lee *et al*[4], Lee *et al*[14], McPhee *et al*[12], Torres *et al*[16], Tenikoff *et al*[17], Wakimoto *et al*[1,32]
	PCSO-524™	PCSO524™, olive oil and D-Alpha-tocopherol.	Kwananocha *et al*[21], Mongkon *et al*[22], Soontornvipart *et al*[23]
	Antinol® (Vetz Petz, Thailand)	GLM, vitamin E, gelatin, glycerine, olive oil	Buddachat *et al*[6]
	SF4-dog (McFarlane Laboratories New Zealand Ltd, Auckland, NZ)	GLM, brewer's yeast, lactose, and tableting aid (magnesium stearate, acacia, and aerosil)	Pollard *et al*[20]

Table 1: **Types of GLM extracts.** Both whole mussel products and lipid-rich extracts are commercially available. Especially Lyprinol® has been studied extensively. Other studies not mentioned in this table have used GLM powder, provided by research facilities.

2.2 Components of GLM

The composition and bioactive components of GLM have been studied and will be described below. Since GLM is a natural product and there are several production processes for GLM extracts, concentrations of biochemical components will be given within a certain range, instead of an absolute concentration.[28]

2.2.1 Biochemical components of GLM

The biochemical components of the whole green lipped mussel have been studied through chemical analysis.[11,28] In one study, GLM was found to contain 40 to 70 g/100g protein, 9,6 to 12 g/100g carbohydrate, and 6 to 15 g/100g lipids.[28] Between 2,8 and 4,5 g/100g of these lipids are the Ω3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) .[28] In another study, the lipid content of three commercially available GLM samples was analyzed. Interestingly, results from this study showed that one of the samples contained very low levels of the possibly bio-active EPA and DHA.[33]

The exact lipid composition of both freeze-dried and frozen green-lipped mussel has also been studied. GLM contains five main lipid classes: sterol esters (SE), triglycerides (TG), free fatty acids (FFA), sterols (ST), and phospholipids (PL).[30] In freeze-dried mussel powder, the total lipid amount was found to be around 8,4%; with TG as the biggest class and FFA as the second biggest class.[30] Several of the components mentioned above are possibly bioactive.

2.2.2 Bioactive components of GLM

It is not completely clear what the bioactive components of GLM are, or how many different bioactive components GLM contains. Bioactivity of both proteins and lipids derived from GLM has been studied. Results from an *in vitro* study showed that proteins isolated from GLM inhibit cyclooxygenase (COX) and thereby the production of inflammatory mediators[10] (for more detail, see the paragraph on *in vitro* studies). In another study, a specific bioactive protein (Pernin) was isolated[34]. In 1993, a study was conducted on the effect of a GLM glycogen extract in rats with experimentally induced OA. It was demonstrated that GLM

administration caused a reduction of footpad size.[35] The researchers suggested this was due to a protein associated with the glycogen, rather than the glycogen itself since bioactivity was absent after the extract was treated with proteinase K.[35] Although some studies are focussed on GLM proteins, there is not much known about the mechanism through which GLM proteins work, and both *in vivo* and *in vitro* studies are needed to clarify this.

More research has been done into the lipid fraction of GLM[2,32]. Both *in vitro* and *in vivo* studies on the activity of crude lipid extracts and lipid classes derived from GLM have been done. A **Ω**-3 polyunsaturated fatty acid (PUFA) from GLM with possible anti-inflammatory activity has been identified.[32] It is suggested that GLM lipids inhibit the production of inflammatory mediators in the arachidonic acid (AA) cascade (prostaglandins and leukotrienes).[2,32] The PUFAs have similar structures to AA and are suspected to act as competitive substrates for COX-1 and -2, and thereby decrease prostaglandin production.[32] PUFAs probably affect the lipoxygenase (LOX) pathway through a comparable mechanism, leading to reduced production of leukotrienes and 5-hydroxyeicosatetraenoic acid (5-HETE).[32,36] It seems likely that lipids from GLM are responsible for (a part) of its anti-inflammatory activity, although the exact mechanism and amount of bioactive lipids in GLM extracts are still unknown. In several *in vitro* studies, researchers chose to focus on either lipid content or protein content. Results from these studies, as well as studies where whole mussel powder has been studied, will be discussed below.

2.3 In vitro studies

Many *in vitro* studies have been previously performed with GLM. Both COX and LOX inhibition assays and *in vitro* cell culture experiments have been performed. In most studies, primary white blood cells[5,9,32], cell lines for research purposes[5,10] or chondrocytes[6,11] are used. Studies are often focused on inflammatory mediator production, and when employing chondrocytes also on specific osteoarthritis parameters.

2.3.1 COX and LOX inhibition assays

It has been proposed that GLM influences the COX/LOX cascade[32,36], therefore the effect of GLM on these enzymes has been studied several times. In all reviewed studies, it was found that compounds from GLM inhibit COX and LOX to some extent, but results varied between studied extracts and concentrations. The summarized results from these studies can be found in table 2.

Mani and Lawson[10] found that inhibition of COX by a protein-rich GLM extract was above 50%. They even found inhibition of 90% for COX-2 when the protein level of the GLM preparation was 25 µg.[10] Inhibition of COX by a glycogen-rich commercial GLM preparation was weaker, but still above 70% for COX-1 and 60% for COX-2 at the highest GLM concentration.[10]

COX inhibition assays have also been performed for several (modified) GLM lipid extracts. A total lipid extract showed only moderate inhibition of COX-1 (12%) and COX-2(25%).[12] Modifications of the lipid extract, such as saponification, hydrolyzation, adding of proteases, and adding of both proteases and lipases, all increased the inhibition of COX.[12] Lipases cleave esterified fatty acids, and thereby increase the concentration of FFAs.[12] The FFA fraction isolated from Lyprinol® caused the strongest inhibition of COX-1 and COX-2.[12] McPhee *et al*[12] suggested that the Lyprinol® inhibited COX as a competitive substrate inhibitor, since incubation of Lyprinol® with COX without the presence of AA still resulted in the production of alternate prostaglandin metabolites.[12]

The inhibition of LOX by lipid fractions from GLM has also been investigated by stimulation of human neutrophils with calcium ionophore, and subsequent measurement of leukotriene B4 (LTB4) and 5-HETE levels.[32] Several separate ω-3 PUFAs isolated from GLM were shown to inhibit LOX[32]. In another study, researchers found that the entire FFA fraction also inhibits LOX.[12] Altogether, results from the three discussed studies seem to indicate that there is a significant effect of the lipid fraction of GLM on the COX/LOX cascade. This is further supported by the influence of GLM on the prostaglandin and leukotriene levels produced by cell lines and primary cells. The effect of GLM on these inflammatory mediators, as well as others, are discussed in the next paragraph.

Compound	Cell type/line	Dose range	Study design	Results	Authors
Tween-20 extract of Perna® and glycogen-rich commercial extract of GLM	Ovine COX-1 and COX-2	100 µl Perna® extract (containing 0 µg, 5 µg, 10 µg, 15 µg, 20 µg, and 25 µg protein)	COX-1 and COX-2 assay with colorimetric Ovine COX assay kit	Inhibition of COX-1 and COX-2 (over 50%), inhibition higher for Tween-20 extract	Mani and Lawson, 2006[10]
ω-3 PUFAs isolated from SFE-CO_2 freeze-dried mussel powder	Human neutrophils	unknown	LOX inhibition assay - stimulation of neutrophils with AA and calcium ionophore.	Inhibition of LOX by several isolated PUFAs, measured through Leukotriene and 5-HETE levels.	Treschow *et al*, 2007[32]
Total lipid extract	Ovine COX-1 and COX-2	1 µg/mL	COX-1 and COX-2 assay - preincubation of test samples with enzyme, room temperature, 5 min	Moderate inhibition of COX-1 and COX-2 (COX-1 12%; COX-2 25%)	McPhee *et al*, 2007[12]
Saponified total lipid extract	Ovine COX-1 and COX-2	1 µg/mL	COX-1 and -2 inhibition assay	Strong inhibition of COX-1 and COX-2 (COX-1, 49%; COX-2, 60%)	McPhee *et al*, 2007[12]
Protease lipid extract and protease-lipase lipid extract	Ovine COX-1 and COX-2	1 µg/mL	COX-1 and -2 inhibition assay	Strong inhibition. Protease lipid extract (COX-1, 57%; COX-2, 47%) and protease–lipase lipid extract (COX-1, 67%; COX-2, 62%).	McPhee *et al*, 2007[12]
Lyprinol®, Hydrolysed Lyprinol®,	Ovine COX-1 and COX-2	1 µg/mL (several concentrations tested)	COX-1 and -2 inhibition assay	Strong inhibition of COX, by Hydrolyzed Lyprinol® (COX-1, 61%; COX-2, 62%). 10 times more effective than non-hydrolyzed	McPhee *et al*, 2007[12]
FFA fraction from Lyprinol®, triglyceride fraction, other lipid fractions	Ovine COX-1 and COX-2	1 µg/mL	COX-1 and -2 inhibition assay.	FFA: Strong inhibition, (COX-1, 78%; COX-2, 70%) Triglyceride: Medium inhibition, (COX-1, 43%; COX-2, 52%) Other lipid fractions: ≤ 32%	McPhee *et al*, 2007[12]

Table 2: **Overview of COX-1/COX-2 and LOX inhibition assays.** Two studies have performed COX-1 and COX-2 inhibition assays. One study used a protein fraction filtered from a Tween-20 Perna® extract and a glycogen-rich commercial extract. COX-1 and COX-2 were both inhibited by over 50% by the protein fraction. McPhee et al used several lipid extracts. The strongest inhibition of COX was found at the FFA fraction from Lyprinol® at a concentration of 1 µg/mL. One LOX inhibition assay with ω-3 PUFAs isolated from GLM has been done, where it was found that several PUFAs inhibit LOX.

2.3.2 Studies with primary cells and cell lines

Anti-inflammatory and immunomodulatory effects of GLM are also assessed through *in vitro* models with primary cells and cell lines. Generally, cells are stimulated to produce inflammatory mediators, and levels between untreated controls and cells treated with GLM are compared. In one study, a full GLM extract was used to test inflammatory mediator production by monocytes[5], in others protein- or lipid-fractions were used. An overview of these studies can be found in tables 3 (whole mussel extracts) and 4 (protein-rich and lipid extracts).

Lawson *et al*[5] found that treatment with Perna® concentrations of 0,1 and 1,0 mg/mL resulted in a decrease in tumor necrosis factor α (TNF-α) and interleukin 12p40 (IL-12p40) production.[5] The influence of a phenol extract of Perna® was assessed by measuring superoxide burst activity of rat neutrophils. All doses decreased burst activity, with the lowest activity point at a concentration of 400 µg/ml (27,4% activity compared to untreated control).[5]

In 2006, an extensive study on Perna® was performed, with a focus on the protein content of two Perna® extracts. One of the measured parameters was Immunoglobulin G (IgG) production by the V2E9 hybrid cell line.[10] The tested HCl extract of Perna® decreased IgG production by 21% at a protein concentration of 20 µg; the Tween-20 extract decreased IgG production by 26% at a concentration of 25 µg (both compared to untreated controls).[10] The decrease in IgG production was dose-dependent, with the highest inhibition at the highest concentrations.[10] HCl extract was not tested above concentrations of 20 µg, probably since HCl was found to negatively affect cell viability in the same study. Several cell lines were incubated with both Perna® extracts. The supernatant was added to responder cell lines to measure cytokine production. Overall, decreased inflammatory mediator production (TNF- α, IL-1, and IL-6) by the Perna® extracts was found, with the largest decrease at a protein concentration of 20 µg of the Tween-20 extract.[10]

In a study from 1997, several lipid fractions of Lyprinol® were tested on human polymorphonuclear leukocytes and human monocytes. Various FFA subfractions were found to inhibit LTB_4, 5-HETE, and prostaglandin E2 (PGE_2) production.[9] A study in 2008 showed similar results, where a ω-3 PUFA from GLM was found to inhibit leukotriene production at a concentration of 24 µg/mL.[15]

The results discussed above, together with the results discussed in the previous paragraph, indicate that the activity of GLM lies at least partially in the COX/LOX cascade. Several other inflammatory mediators are influenced as well, but it is not yet clear through which mechanism.

Compound	Cell type/line	Dose range	Study design	Parameters measured	Results	Authors
Whole mussel extracts						
Tween-20 extract of Perna®	Human THP-1 monocytes (differentiated into mature)	0, 0,0001, 0,001, 0,01, 0,1, 1,0 mg/ml	- primed with recombinant INF-γ (10 ng/ml) for 16hrs - Preincubated for 2 hrs with and without Perna® extract - stimulation with LPS (1 µg/ml)	IL-12p40 TNF-α	Dose-dependent reduction of cytokine production, significant at Perna® concentrations of 0,1 and 1,0 mg/ml	Lawson *et al*, 2007[5]
Phenol extract of Perna®	Rat neutrophils	0, 100, 200 and 400 µg/ml	- Stimulation with PMA (50 ng/ml)	Superoxides (an indicator of neutrophil burst activity)	Dose-dependent reduction of superoxides, at all Perna® concentrations.	Lawson *et al*, 2007[5]

Table 3: **Overview of *in vitro* studies with whole mussel extracts.** IL-12p40 and TNF-α were both reduced by Perna® concentrations of 0.1 and 1.0 mg/ml. Superoxide production was inhibited by all tested Perna® concentrations.

Compound	Cell type/line	Dose range	Study design	Parameters	Results	Authors
GLM with a focus on Protein fraction						
Hydrochloric acid extract of Perna® and Tween-20 extract of Perna®	V2E9	HCL: 10, 15, and 20 µg protein Tween 20: 100 µl Perna® extract (containing 0 µg, 5 µg, 10 µg, 15 µg, 20 µg, and 25 µg protein)	- Incubation at 37°C for 48 hours - Extract filtered through 0.22 µ filter, protein content estimated - Measurement of IgG - Treatment of Tween-20 with proteinase-K, to degrade proteins	IgG	HCl extract: decrease in IgG levels of 21% at 20 µg [protein] Tween-20: decrease in IgG levels of 26% at 25 µg [protein] Tween-20 with proteinase-K: no decrease in IgG	Mani and Lawson, 2006[10]
Hydrochloric acid extract of Perna® and Tween-20 extract of Perna®	THP-1 and L-929 U-937 and A375.S2 Jurkat E6-1 and CTLL-2 + HT-2 EL-4 and CTLL-2 + HT-2 LS 174T and 7TD1	As above	- THP-1 stimulated with LPS - others with Ionomycin and PMA - Supernatant added to responder cell-line - Amount of responder cell-lines measured - rest as described above	TNF- α IL-1 IL-2 IL-6	In general, a decrease of cytokine secretion compared to controls. Largest decrease at 20 µg of Tween-20 extract	Mani and Lawson, 2006[10]
Lipid fraction						
Lyprinol® and subfractions	Human polymorphonuclear leukocytes	Lyprinol®: 100 µg/ml, subfractions: range from 9 to 155 µg/ml	AA added (10 µmol/L) Stimulation with calcium ionophore (5 µmol/L)	LTB4	Inhibition of LTB_4 and 5-HETE by 4 subfractions	Whitehouse *et al*, 1997[9]
Lyprinol® and subfractions	Human monocytes	As above	Challenged with LPS No pre-incubation	PGE_2	Inhibition of PGE_2 production	Whitehouse *et al*, 1997[9]
ω-3 PUFA from freeze-dried mussel powder	Human neutrophils	Several doses, working dose 24 µg/mL	Inhibition of leukotriene biosynthesis Stimulation with calcium ionophore	LTB4, 5-HETE, and two non-enzymic isomers, 6-trans LTB4 and 6-trans, 12-epi LTB4.	Inhibition of leukotriene production between 35–70%	Singh *et al*, 2008[15]

Table 4: **Overview of *in vitro* studies with protein-rich extracts and lipid extracts.** One study used protein fractions from Tween-20 and hydrochloric Perna® extracts. IgG, TNF-α, IL-1, IL-2, and IL-6 concentrations in medium all decreased compared to controls. Treatment of Tween-20 Perna® extract with proteinase-A resulted in no decrease in IgG levels compared to controls, supporting the fact that proteins are responsible for the decrease in IgG levels.

2.3.3 Studies with cartilage explants and chondrocytes

In two studies, researchers have used cartilage explants to analyze the effect of GLM on arthritic conditions. It was found that Antinol® was able to counteract the effect of IL-1β on gene expression in canine chondrocytes.[6] Antinol® was able to downregulate the expression of the TNF-α gene but did not directly affect the expression of the IL-1β gene.[6] In another study, it was found that without IL-1 present, conditioning of cartilage explants with dehydrated GLM powder was not able to decrease PGE_2 production. Nevertheless, when porcine cartilage was stimulated with IL-1, GLM did downregulate PGE_2 production.[11] The results from these studies might suggest that GLM actively counteracts IL-1.[11]

As mentioned above, these two studies mostly focused on parameters of arthritic conditions. In both studies, it was found that GLM (Antinol® in one study[6] and whole mussel powder in the other[11]) protects against matrix degradation of cartilage, by preventing the release of glycosaminoglycans (GAGs).[6,11] GLM did not prevent uronic acid and hydroxyproline loss[6], nor did it prevent an IL-1 induced increase in nitric oxide (NO) production[11].

Altogether, the results from these *in vitro* studies indicate that GLM extracts affect the production of inflammatory mediators from the COX/LOX cascade (such as PGE_2, 5-HETE, and LTB_4) and others such as TNF-α and interleukins.

2.4 In vivo studies

Most *in vivo* studies in laboratory animals (rats and mice) and veterinary patients (mostly dogs) focus on clinical OA parameters. However, in some studies, this is combined with an analysis of inflammatory mediator production.

In several studies with laboratory animals and veterinary patients, Lyprinol® or fatty acid compounds derived from whole mussel powder were used as the test compound. In two studies, inflammatory mediator production was evaluated by inducing arthritis in Sprague-Dawley rats and subsequently treating them with Lyprinol® at a dose of 25 mg/kg bw/day. After treatment of 14 and 28 days, splenocytes were extracted from the rats and inflammatory mediator levels were measured following LPS stimulation.[4,13] In both of these studies, a decrease in TNF-α and interferon γ (INF-γ) production by the extracted splenocytes was observed.[4,13] Interestingly, in the second study, there was no observed difference in clinical arthritis scores between treated rats and control rats.[13] However, in a study focused on allergic airway disease, induced with ovalbumin in mice, it was found that although Lyprinol® was effective in reducing airway hyperresponsiveness, it did not influence the levels of IL-4, IL-5, IL-13, and INF-γ produced by cells extracted from peribronchial lymph nodes.[37]

In other studies, it was found that treatment with several lipid extracts from GLM significantly reduced paw swelling[1,9,15] and arthritic score[9] in OA rat models. However, treatment generally had no short-term effect, and significant differences were only seen after prolonged treatment (in one study after 15 days).[9,15] Studies with PCSO-524™ (a fatty acid compound of GLM) in dogs suffering from OA produced similar results; in general, after 4 weeks of treatment, clinical lameness scores improved.[21-23] In one study, collagen-induced arthritis in rats was treated with a combination of Lyprinol® and meloxicam. Researchers found the treatment of rats with both compounds, increased the effectivity of meloxicam on the reduction of arthritic scores.[38]

There are also several studies in which the effect of whole mussel powder is studied. In one study, the effect of GLM on serum levels of IL-2, IgM, and IgG was evaluated in rats with collagen-induced arthritis. Only a decrease in anti-collagen IgM production was detected; IL-2 and IgG levels were not affected.[5] In the same study, it was found that Perna® had no effect on initial arthritic scores in mice, but after 107 days a reduction of arthritic scores was observed.[5] In two other studies, the effectiveness of freeze-dried, whole mussel powder in canine OA patients was evaluated. A difference in total arthritic scores was observed, but again only after prolonged treatment (6 weeks).[18,19] In another study, freeze-dried whole mussel powder was tested in dogs suffering from OA, measured through lameness and pain as interpreted by their owners. Owners observed no difference after a 12-week treatment with a dose of 11 mg/kg bw/day.[25] The only study where GLM was tested in horses, equine patients suffering from chronic fetlock lameness due to OA were treated with Biolane®.[24] It was found that Biolane® was able to significantly reduce lameness severity and joint pain post-treatment (the duration of treatment was generally 56 days).[24]

In conclusion, it can be stated that the *in vivo* effects of GLM on serum interleukins and immunoglobulins is less pronounced than its effect *in vitro,* and often completely absent. However, when splenocytes are extracted after GLM treatment, a decrease in inflammatory mediator production has been observed twice. *In vivo*, the effect of GLM on inflammatory mediators is not evident, however, clinical improvement of OA patients is often observed after prolonged treatment with GLM.

2.5 Aim and objective of this study

Altogether, results from *in vitro* studies indicate that GLM has anti-inflammatory properties, without being toxic to cells. The whole mussel powder was found to inhibit IL-12p40 and TNF-α production by Human THP-1 monocytes at concentrations of 1.0 and 0.1 mg/ml[5], as well as PGE_2 production in porcine cartilage explants[11]. The expression of the TNF-α gene in canine chondrocytes was influenced by whole mussel powder, but it did not influence the expression of the IL-1β gene.[6] In one study, high concentrations of Perna® inhibited COX-1 and COX-2 by over 50% and decreased IgG, TNF- α, IL-1, IL-2, and IL-6 production by several cell lines.[10] In two other studies, lipid extracts from GLM inhibited COX-1 and COX-2 most effectively at lipid concentrations of 1 μg/mL[12] and inhibited PGE_2 production by human monocytes[9]. Results from *in vivo* studies in rats, mice, and dogs also are promising. GLM treatment was found to improve clinical lameness[21-23], paw swelling[1,9,15], and arthritic score[9,19,29], yet effects on inflammatory mediators are often absent.

Both *in vitro* and *in vivo* effects of GLM in equine models are largely unknown at the moment, but studies using other animals and cells indicate that GLM might have beneficial effects in horses as well. The aim of this study is to examine the possible anti-inflammatory properties of GLM in an equine model. Therefore, we performed a study into the anti-inflammatory effects of a green-lipped mussel extract in LPS-challenged equine PBMCs *ex vivo*. Moreover, these effects were compared with the anti-inflammatory effects of the well-established NSAID meloxicam. Before the anti-inflammatory properties of GLM were studied, cell-toxicity of GLM for equine PBMCs was studied. Hereafter, equine PBMCs were incubated with GLM, meloxicam, or the solvent of the GLM extract. Meloxicam is often used in the treatment of lameness and has proven efficacy in horses.[39,40] *In vitro* studies with meloxicam have shown that it is a strong COX inhibitor in equine (whole) blood[41,42], and therefore, served as a positive control. The incubations were performed for both LPS-challenged and unchallenged cells. To assess anti-inflammatory properties, levels of TNF-α and PGE_2 were measured. These levels were compared between GLM and untreated controls, as well as between GLM and meloxicam.

3. Materials and methods

Methods for sample collection, PMBC isolation, cytotoxicity assay, and cell culture experiments were based on previously performed research.[7]

3.1 Literature study

Before starting the experiments, a literature study on GLM was conducted. Databases used to find relevant literature were PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) and Google Scholar (https://scholar.google.nl/). Several keywords were used, some of them separately, but most often combined: green lipped mussel, GLM, *Perna canaliculus*, meloxicam, immunomodulatory, anti-inflammatory, cytokines, COX, LOX, osteoarthritis, synovitis, in vitro, in vivo, horse, equine, veterinary patient, treatment. Relevant literature cited in other studies and reviews was also used.

3.2 Solutions

The cell culture medium was prepared by combining 89% RPMI 1640 Medium (1X) + GlutaMAX™ (Gibco Life Technologies, 61870-010), 10% horse serum (Invitrogen, 31874) and 1% Penicillin (100IU/ml)/ Streptomycin (100µg/ml) (BioWhittaker, DE 17-602E).

PBS/EDTA stock solution (100 mM) was made by dissolving 3722,4 mg EDTA disodium salt, with a molecular weight of 372,24 g/mole, in 100 mL Phosphate Buffered Saline without Ca/Mg (Gibco Life Technologies, 14190). The stock solution was filtered through a 0.2 µM filter and stored at -20 °C until it was needed. To acquire the working solution, the PBS/EDTA stock solution was diluted with PBS, to reach a concentration of 2 mM.

The GLMax® and the solvent stock solutions were kindly provided by Synofit Europe bv. The GLMax® solution contains liquid, 3,5% GLMax® (35 mg/ml), 2% lactic acid, 0,4% citric acid and 0,3% potassium sorbate. The solvent of GLMax® has the same composition, excluding GLMax®. The meloxicam solution was made by dissolving Meloxicam sodium salt (Bio Connect life sciences, sc-215294) in the cell culture medium, to reach the highest working concentration of 0,1 mg/ml. Dilutions used in the experiments were based on an estimated plasma concentration in live horses. Based on the dosing advice by Synopet for GLMax® (15 ml/day, containing 525 mg GLM), the estimated maximum plasma concentration was 0,00875 mg/mL. The plasma concentration of meloxicam after single administration was estimated at 0,0188 mg/mL, based on a study on meloxicam plasma levels[8]. Dilutions in cell culture experiments were near these concentrations, as well as 10 times higher and lower. All solutions were filtered through a 0,2 µm filter, to prevent large particles from disturbing the experiments.

3.3 Animals and sample collection

Blood samples were taken by jugular venipuncture from 4 healthy warmblood horses and placed in tubes containing 20 IU heparin/ml blood. Samples were kept at room temperature and processed within 30 minutes after collection.

3.4 PMBC isolation

Density gradient media, Lymphoprep™ (Stemcell technologies, #07801) or Ficoll Paque® (GE Life sciences, 17-1440-02), were brought to room temperature. The obtained blood was diluted by adding PBS/EDTA 2mM in a 1:1 ratio. The diluted blood was layered onto the density gradient medium in 50 mL plastic centrifuge tubes. The tubes were centrifuged at 400 x g for 30 minutes at 21 ^{0}C, without applying the brakes. The buffy coat was pipetted into new 50 ml plastic centrifuge tubes. Cells were washed twice, by diluting the buffy coat with warmed PBS, and centrifuged at 400 x g for 15 minutes to remove contaminations and platelets. The cells were re-suspended in fully supplemented cell culture medium (as described above) and cells were counted after making a 1:10 dilution with Trypan blue. After cell count, PBMCs were further diluted in cell culture medium to reach a seeding density of 4 x 10^6 cells/ml and were incubated in a glass bottle at 4 ^{0}C overnight. Experiments were performed the following day.

3.5 Cell culture experiments

Equine PBMCs were seeded at a density of $4*10^6$ cells/ml/well in 24 well plates and the plates were placed in an incubator at 37 °C and 5% CO_2/95% air, while dilutions series were made. Cell culture experiments were performed for GLMax®, meloxicam (as a positive control), and the solvent. Three dilutions for every compound were tested, with a concentration of 0,1, 0,01 and 0,001 mg/ml of every agent.
In the third experiment, a concentration of 0,0001 mg/ml was added for every compound. GLMax® and solvent (from the 35 mg/ml stock) were pipetted into medium and filtered through a 0,2 µm filter. Meloxicam was weighed and directly added to the cell culture medium, and filtered through a 0,2 µm filter. PBMCs were taken out of the incubator and centrifuged for 10 minutes at 400 x g to spin PBMCs to the bottom of the wells. Subsequently, the cell culture medium was removed and the cells were incubated according to the protocol below.

First, the PBMCs were pre-incubated with GLMax®, meloxicam, and solvent concentrations. Negative controls were included as well. Cells were pre-incubated for 2 hours at 37 °C and 5% CO_2/95% air. After 2 hours, the medium was pipetted off. Plates were incubated either with the same test compound or blank medium. The cells were challenged with LPS at a concentration of 100 ng/ml or left unchallenged. For the plate setups, see Appendices A and B. All plates were incubated for 4 hours at 37 °C and 5% CO_2/95% air. After incubation, supernatants were collected and samples were stored at -80 °C. After collection supernatant collection, the remaining supernatant was aspirated off and cells were lysed with lysis buffer. Lysis buffer was prepared by adding 60 mL RNA lysis buffer to 1,2 ml β-mercaptoethanol, both from the SV Total RNA Isolation System (Promega Corporation, Z3101). Lysed cells from every well were frozen in 1,5 mL tubes and stored at -80°C to preserve for further analysis.

Plate	Pre-incubation	Incubation
1	GLMax®	GLMax® + LPS or medium + LPS
2	GLMax®	GLMax® or blank medium
3	Solvent	Solvent + LPS or medium + LPS
4	Solvent	Solvent or blank medium
5	Meloxicam	Meloxicam + LPS or medium + LPS
6	Meloxicam	Meloxicam or blank medium

Table 5: **Pre-incubation and incubation schedule.** PBMCs were pre-incubated with test compounds as described above. Incubation was either with test compound + LPS, medium + LPS, test compound, or blank medium. For the exact plate setup, see appendix A.

3.6 Cytotoxicity assay

Cytotoxicity was assessed for GLMax®, Meloxicam, and the solvent solution. First, dilution series for all three solutions were made. GLMax® or solvent solution (35 mg/ml stock) was pipetted into cell culture medium and filtered through a 0,2 µm filter. Meloxicam was directly added to 3500 µL medium and filtered through a 0,2 µm filter. Dilution series were made by diluting every solution 1:1 for 10 dilutions, resulting in concentrations ranging from 0,001953125 mg/ml – 1,0 mg/ml. PBMCs were seeded in three 96 well plates, at a density $8*10^5$ cells per well; cells were not seeded in the outer edges of the plate. The plates were incubated for 2 hours at 37°C and 5% CO_2/95% air. The medium was pipetted off and fresh medium containing the various concentrations of GLMax®, meloxicam, and solvent were added. Alamarblue™ (ThermoFisher Scientific, DAL1025) was added, and cells were incubated for a total of 6 hours. Fluorescence (590 nm) and absorbance (at 570nm and 600nm) were measured every hour.

Similar cytotoxicity assays were also performed simultaneously with the cell culture experiments. PBMCs were seeded in 96 well plates, at a density of $8*10^5$ cells per well. The plates were incubated with 200 µL of the working concentrations of test compounds as well as 20 µL Alamarblue™. Fluorescence (590 nm) and absorbance (at 570nm and 600nm) were measured after 4 and 6 hours.

3.7 TNF-α ELISA

Levels of TNF-α produced by equine PBMCs during the cell culture experiments were measured in supernatants utilizing the Equine TNF-alpha DuoSet Enzyme-Linked Immunosorbent Assay (ELISA) (R&D systems, DY1814). ELISAs were performed according to the manufacturer's instructions. The standard had a lower detection limit of 31,3 pg/ml, and an upper detection limit of 2000 pg/ml. Supernatants from LPS-challenged cells were diluted 10x and 100x with Reagent Diluent (R&D systems, DY995). Supernatants from unchallenged cells were not diluted. All concentrations in the undiluted samples were low enough to be interpolated in the calibration graph. Measurements were done for all undiluted samples, as well as the 10x and 100x diluted samples.

3.8 HPLC

PGE_2 concentrations in supernatants were measured through high-performance liquid chromatography (HPLC). First, a standard curve of PGmix was prepared, ranging from 6.25 pg/µl to 200 pg/µl. PGE_2 was then extracted from the supernatant. 0,2M NH_4-COOH pH 3,3, ethyl acetate, and internal standard (IS) were added to the samples, which were then centrifuged and frozen. The non-frozen layer was pipetted off and 0,2M NH_4-COOH pH 3,3 and ethyl acetate were added again. The samples were vortexed and the fluid was evaporated in a Speedvac. After evaporation, methanol was added, and samples were pipetted onto a 96-well plate. Samples were stored at -80 °C until analysis could be performed. All samples were later measured with HPLC, and total area measurements for PGE_2 and IS were transferred to excel.

3.9 Calculations and statistics

Data collected during the TNF-α ELISAs were interpolated using the standard curve measurements. A standard curve was plotted using Graphpad Prism v.7 by doing a logistic transformation of the data followed by a 4 parameter logistic curve fit. Measurement data were interpolated and the reverse log was used to obtain the correct concentrations. If applicable, the calculated concentrations were multiplied by their dilution factor. The percentages mentioned in the results section were calculated as (mean result of the replicates/mean negative control)*100.

Data from HPLC measurements were used to calculate PGE_2 concentrations in Excel v.2013. First, the area under the peak measurements from the PGE_2 standard curve were corrected with the area under the peak from the internal standard measurements. These corrected measurements were plotted and a regression line was fitted to the data. The unknown concentrations were calculated by using the intercept and slope of the regression line.

In the second and third experiments, cytotoxicity was also measured. The percentage of viable cells was calculated following the manufacturer's instructions, with the following formula:

$$\%\ viability = \frac{A_{lw} - (A_{hw} \times R_0)\ test\ well}{A_{lw} - (A_{hw} \times R_0)\ positive\ growth\ control} \times 100.$$

Where A_{lw} = absorbance at the low wavelength, A_{hw} = absorbance at the high wavelength, and R_0 = correction factor. The correction factor was calculated with the following formula:

$$R_0 = \frac{AO_{lw}}{AO_{hw}}$$

Where AO_{lw} = absorbance of AlamarBlue™ in medium - absorbance of medium only (at lower wavelength) and AO_{hw} = absorbance of AlamarBlue™ in medium - absorbance of medium only (at higher wavelength).

Statistics on all data were performed in SPSS v.24. Data from the three cell culture experiments were analyzed separately since cells donated by different horses showed different reactivity strength. For every experiment, Levene's test for homogeneity was performed, followed by a one-way ANOVA and Tukey's post hoc test. All α values were set at $p \leq 0.05$. For data sets where Levene's test for homogeneity was significant, a log-transformation was performed, followed by a second Levene's test, one-way ANOVA, and Tukey's post hoc test.

4. Results

4.1 TNF-α production by equine PBMCS

The results for every experiment are discussed separately, due to differences in reactivity to LPS and the test compounds of different donors. This effect has been observed previously by Vendrig *et al.*[8] Every experiment contained three replicates of every treatment, the results are discussed as the mean of every replicate.

4.1.1 TNF-α production after LPS challenge

LPS-challenged equine PBMCs all showed a significant increase in TNF-α production, compared to unchallenged PBMCs (results from unchallenged PBMCs not shown). In the third experiment, the reactivity of PBMCs was tested with an LPS concentration range. The results can be found in figure 1. On plate 3, a slight, but significant increase of TNF-α was found for a concentration of 0,1 ng/ml LPS. All concentrations above also significantly increased TNF-α levels, compared to unchallenged PBMCs. However, there was no significant difference between LPS concentrations of 10 and 100 ng/ml. On plate 9, a significant increase of TNF-α compared to 0 was found at LPS concentrations of 1, 10, and 100 ng/ml. Again, there was no significant difference between TNF-α levels of cells challenged with 10 and 100 ng/ml. The variance of plate 6 was too high to use a one-way ANOVA, but data followed the same trend as those from plate 3.

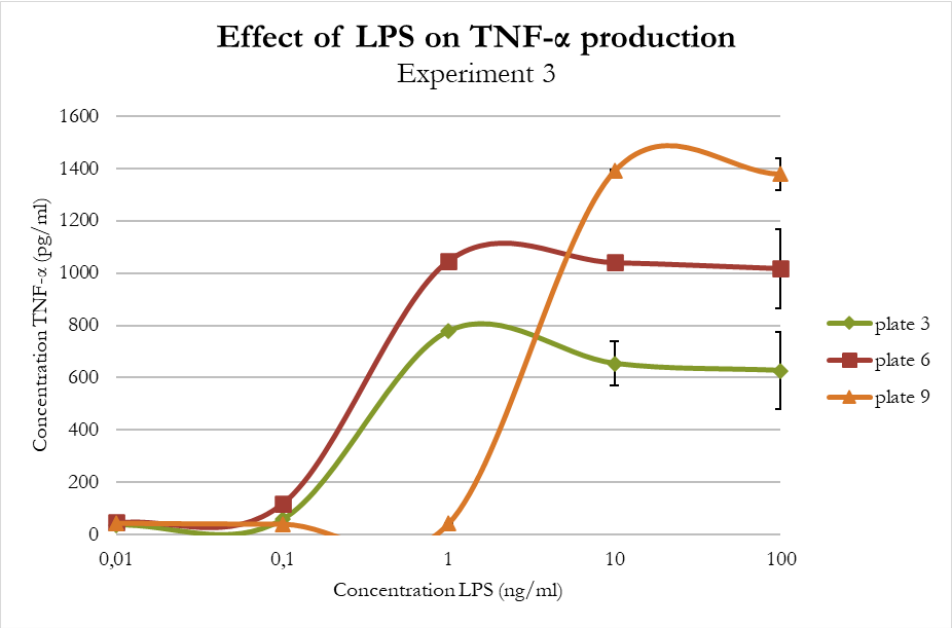


Figure 1: **TNF-α levels produced by equine PBMCs, after pre-incubation blank medium and incubation with LPS.** TNF-α was measured with ELISA in supernatants collected after the cell culture experiment. The datapoints display mean TNF-α levels of three replicates. Error bars display +/- 1 standard deviation.

4.1.1.3 Effect of GLMax®

The effect of GLMax® on the TNF-α production by LPS-challenged PBMCs was studied in three experiments. As described in materials and methods, cells were incubated with GLMax®, and subsequently challenged with LPS. The graphs in figures 2 and 3 show the results from these experiments. In the first experiment, GLMax® at a concentration of 0,1 mg/ml was found to significantly reduce TNF-α levels after pre-incubation with GLMax® and incubation with LPS. The mean TNF-α level at this concentration was 22,20% lower compared to the negative control.

Other concentrations did not significantly influence TNF-α levels after pre-incubation. GLMax® was also found to significantly reduce TNF-α levels after incubation, at a concentration of 0,001 mg/ml (17,23% reduction). Other GLMax® concentrations did not significantly influence TNF-α levels after incubation. In the second experiment, GLMax® at a concentration of 0,001 mg/ml and 0,01 mg/ml significantly reduced TNF-α levels after pre-incubation (respectively 45,14% and 38,86%). After incubation with GLMax®, the TNF- α level for the GLMax® concentration of 0,001 mg/ml was significantly reduced with 36,81%. Other concentrations in the second experiment produced no significant results. Interestingly, there was no significant observed effect of GLMax® on LPS-challenged cells in the third experiment.

4.1.1.2 Effect of meloxicam

In all experiments, the effect of meloxicam on TNF-α levels produced by LPS-challenged equine PBMCs was measured. In the first experiment, a slight, but significant increase of TNF-α compared to negative controls was found after pre-incubation with 0,01 mg/ml meloxicam was found (3,19%). After incubation with meloxicam, the concentration of 0,1 mg/ml caused a significant 20,5% increase in the TNF-α level, but concentrations of 0,01 mg/ml and 0,001 mg/ml caused a significant decrease in TNF-α levels (respectively 11,05 and 12,65%). In the second experiment, concentrations of 0,001 and 0,1 mg/ml significantly reduced TNF-α levels after pre-incubation with 42% and 44% compared to negative controls. In the third experiment, meloxicam had no significant effect.

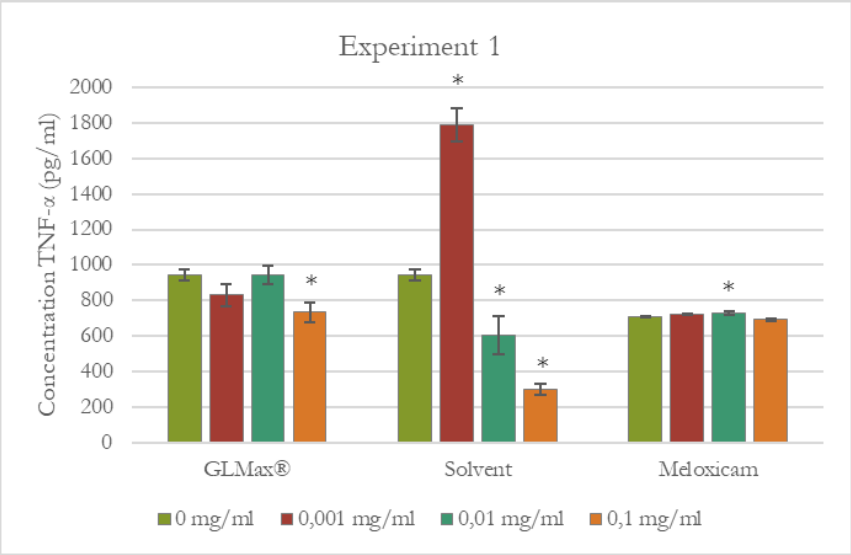
4.1.1.4 Effect of solvent

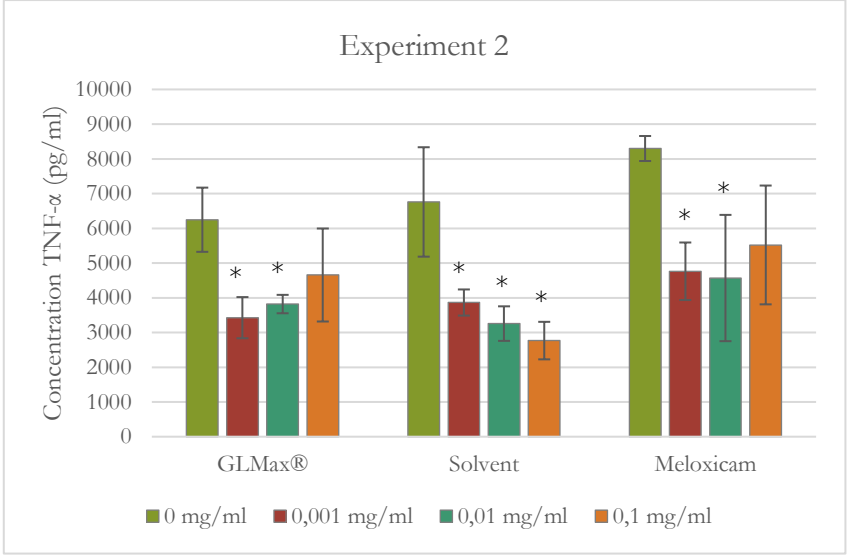
The solvent solution was tested alongside the other test compounds, in the same dilution series as GLMax®. In the first experiment, the solvent solution at a concentration of 0,001 mg/ml caused a significant increase in TNF-α levels; 89.89% increase after pre-incubation and 59,93% after incubation. Both other concentrations caused a significant decrease in TNF-α levels, with the lowest point after pre-incubation with 0,1 mg/ml (68,10% reduction). In the second experiment, all solvent concentrations reduced the levels of TNF-α, both after pre-incubation and incubation. A concentration of 0,1 mg/ml at pre-incubation showed the strongest inhibition, with a 59.06% reduction of TNF-α compared to negative controls. In the third experiment, the only significant effect observed was a 56,06% increase of TNF-α after pre-incubation with 0,0001 mg/ml.

4.1.2 TNF-α production without LPS challenge

During all three experiments, it was found several times that the presence of GLMax® or the solvent solution caused an increase in TNF-α levels in the absence of LPS (results not displayed). Especially the higher test concentrations showed this effect. Incubation with GLMax® at a concentration of 0,1 mg/ml caused a significant increase in TNF-α levels in all three experiments. In the second experiment, the TNF-α level was as much as 10 times higher than the negative control. Pre-incubation with 0,1 mg/ml GLMax® caused an increase in TNF-α in the first and second experiment, with a rise from 69,77 pg/ml to 201,95 pg/ml. The same concentration (0,1 mg/ml) of the solvent solution caused significant increases in TNF-α levels in all experiments, after incubation. After pre-incubation a significant increase was observed in the first and second experiments; an increase was observed in the third experiment as well but significance could not be proven due to high variance. The biggest increase in the second experiment after incubation, where a mean TNF-α level of 426,04 pg/ml was observed (compared to 63,92 pg/ml in the negative control). Solvent concentrations of 0,01 mg/ml also caused a significant increase in the second experiment after both pre-incubation and incubation, and in the third experiment after incubation. Meloxicam generally produced no significant effect on unchallenged cells, except in the first experiment, where there was a slight but significant increase observed after incubation with 0,001 mg/ml.

Figure 2: TNF-α levels produced by equine PBMCs, after pre-incubation with GLMax®, Solvent or Meloxicam and incubation with LPS.





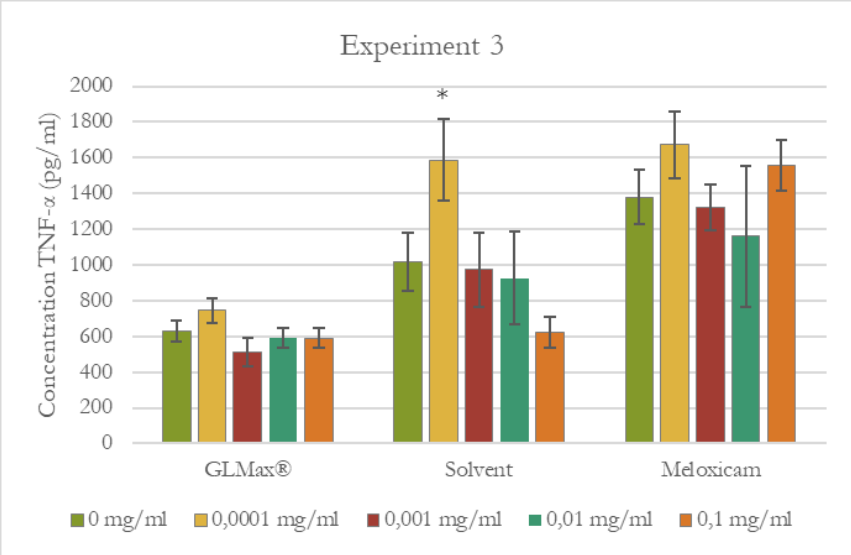
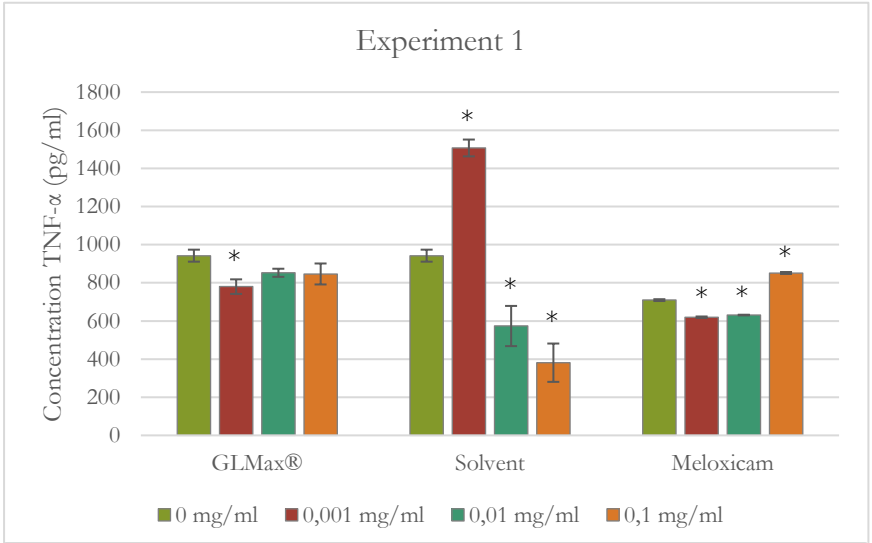
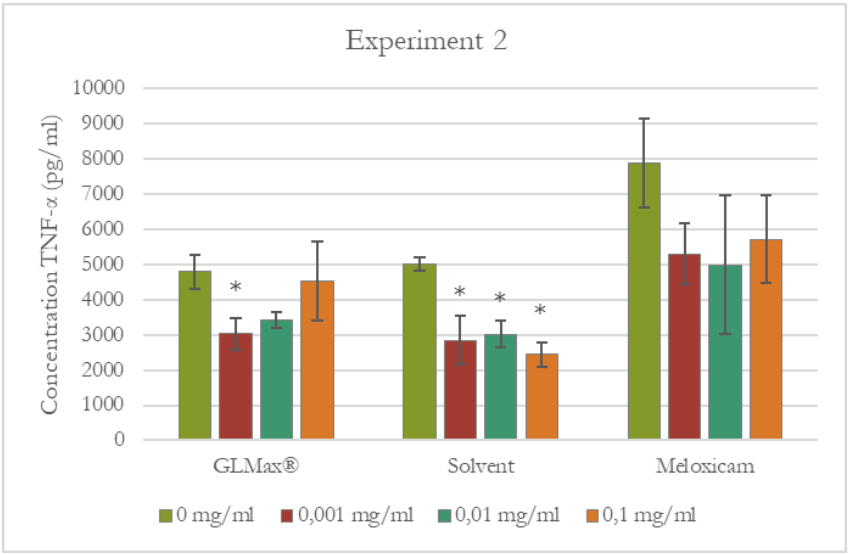


***Figure 2:* TNF-α levels produced by equine PBMCs, after pre-incubation with GLMax®, Solvent or Meloxicam and incubation with LPS.** TNF-α levels were determined with ELISA in supernatants collected after the cell culture experiments. Bars display mean TNF-α levels of three replicates. Error bars display +/- 1 standard deviation. * displays a significant difference with the negative control.

Figure 3: TNF-α levels produced by equine PBMCs, after pre-incubation with GLMax®, Solvent or Meloxicam and incubation with GLMax®, Solvent or Meloxicam, and LPS





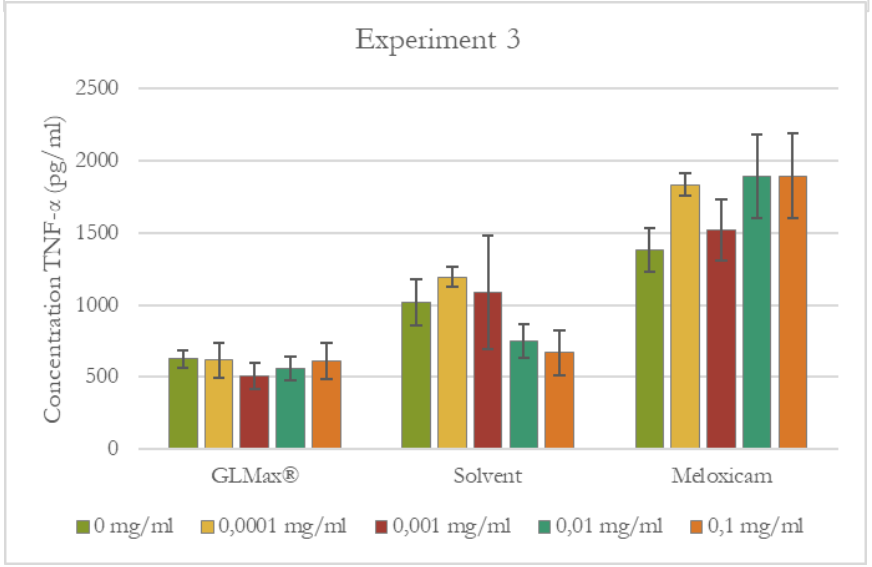
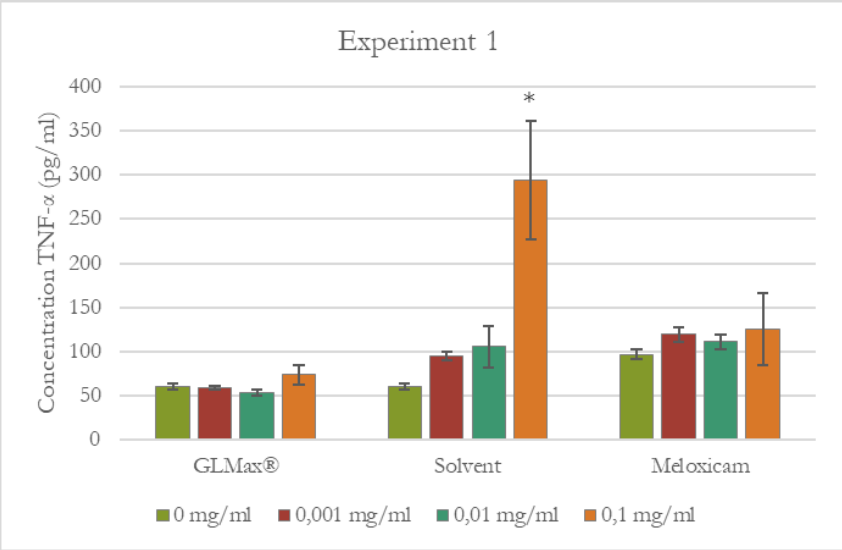
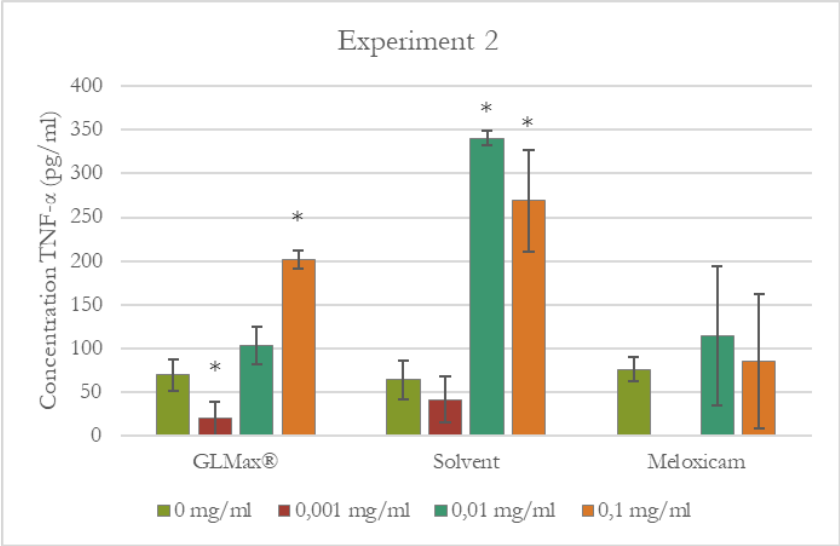


Figure 3: **TNF-α levels produced by equine PBMCs, after pre-incubation with GLMax®, Solvent or Meloxicam and incubation with GLMax®, Solvent or Meloxicam, and LPS.** TNF-α levels were determined with ELISA in supernatants collected after the cell culture experiments. Bars display mean TNF-α levels of three replicates. Error bars display +/- 1 standard deviation. * displays a significant difference with the negative control.

Figure 4: TNF-α levels produced by equine PBMCs, after pre-incubation with GLMax®, Solvent or Meloxicam and incubation with blank medium





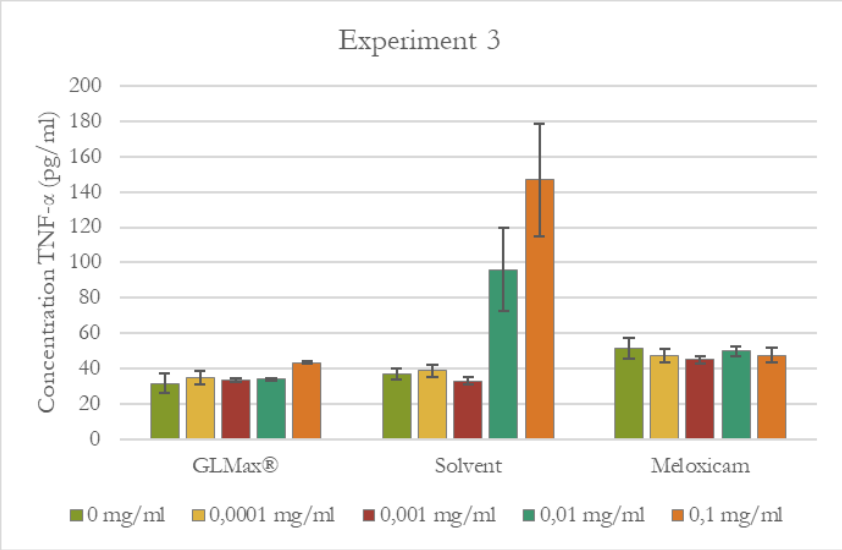
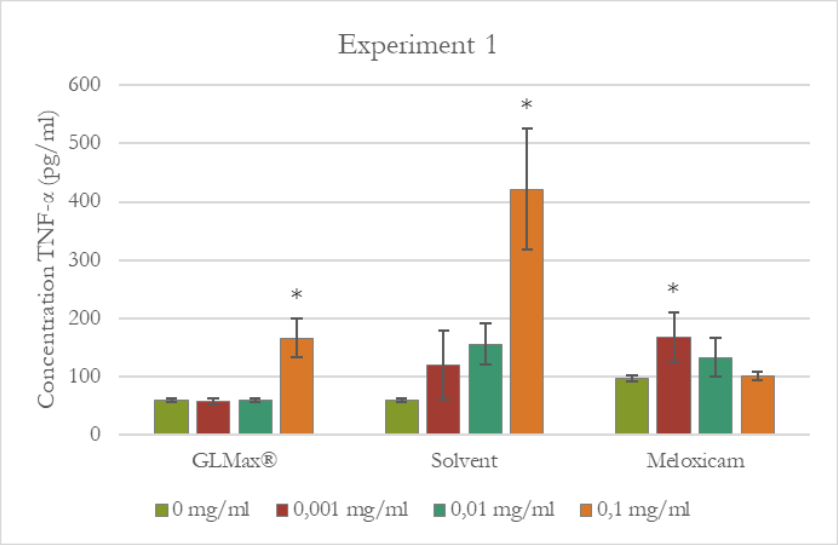
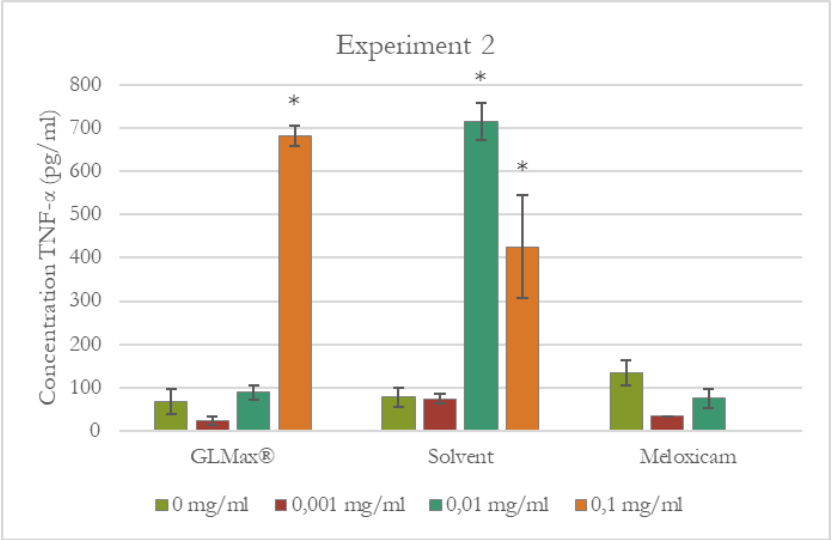


Figure 4: **TNF-α levels produced by equine PBMCs, after pre-incubation with GLMax®, Solvent or Meloxicam and incubation with blank medium.** TNF-α levels were determined with ELISA in supernatants collected after the cell culture experiments. Bars display mean TNF-α levels of three replicates. Error bars display +/- 1 standard deviation. * displays a significant difference with the negative control.

Figure 5: TNF-α levels produced by equine PBMCs, after pre-incubation with GLMax®, Solvent, or Meloxicam and incubation with the same components





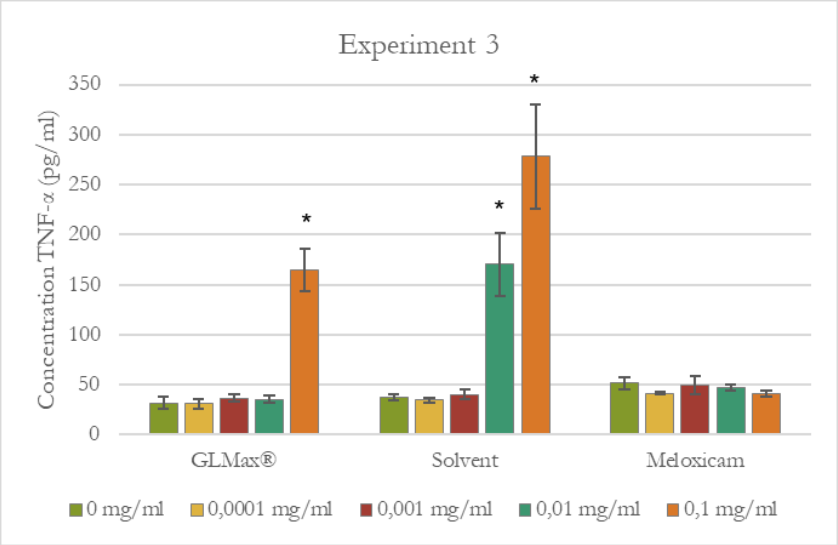


Figure 5: **TNF-α levels produced by equine PBMCs, after pre-incubation with GLMax®, Solvent or Meloxicam and incubation with the same components.** TNF-α levels were determined with ELISA in supernatants collected after the cell culture experiments. Bars display mean TNF-α levels of three replicates. Error bars display +/- 1 standard deviation. * displays a significant difference with the negative control.

4.2 PGE_2 production by equine PBMCS

PGE_2 levels in the supernatants from all three experiments were measured by HPLC as described in materials and methods. Measurements of PGE_2 in supernatants were only done for cells that were treated with the highest concentration of GLMax®, meloxicam, and the solvent (0,1 mg/ml). Only replicates pre-incubated and incubated with the test compounds, and incubated with LPS were used for PGE_2 measurements. Cells only incubated with blank medium were also included. Again, results from the three experiments are discussed separately because of the variation in the reactivity of cells originating from different donors. The results are displayed in figure 6.

4.2.1 PGE_2 production after LPS challenge

The levels of PGE_2 produced by equine PBMCs after LPS challenge was not tested separately from the cell-culture experiments as it was done for TNF-α. However, results from cells incubated with blank medium can be compared to results from cells challenged with LPS. In the first and third experiments, PGE_2 levels were slightly higher in supernatants from challenged cells. In the second experiment, unchallenged cells even produced slightly higher levels of PGE_2 compared to LPS challenged cells. In none of the experiments, a significant difference in PGE_2 levels between unchallenged and LPS challenged cells could not be demonstrated.

4.2.1.1 Effect of GLMax®

As mentioned above, only the effect on PGE_2 after pre-incubation with 0.1 mg/ml GLMax® and incubation with 0.1 mg/ml GLMax® and LPS was measured. In all three experiments, PGE_2 levels after incubation with GLMax® were significantly higher than cells only challenged with LPS. Figure 6 shows the drastic increase in PGE_2 concentrations after incubation with GLMax®. In the first experiment, PGE_2 levels increased by 367,77% compared to the LPS challenged cells. In the second experiment a 382,18% increase was found and in the third a 325,51% increase.

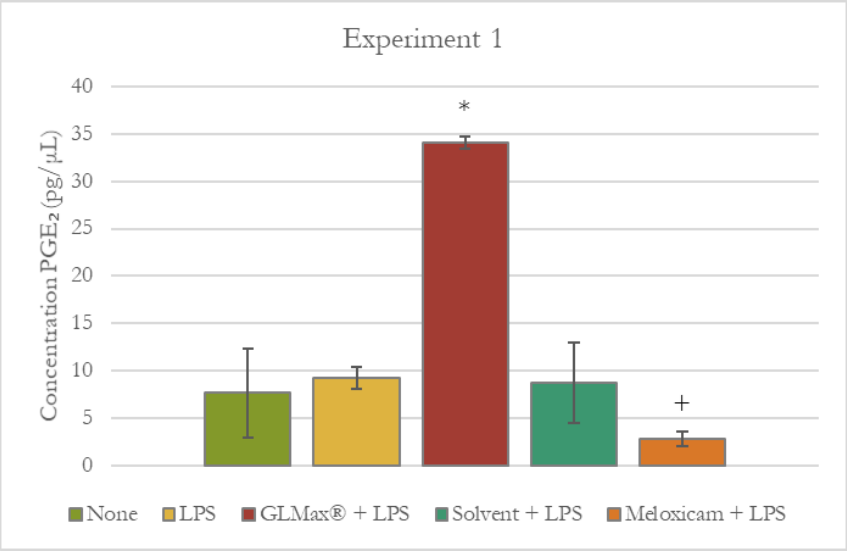
4.2.1.2 Effect of meloxicam

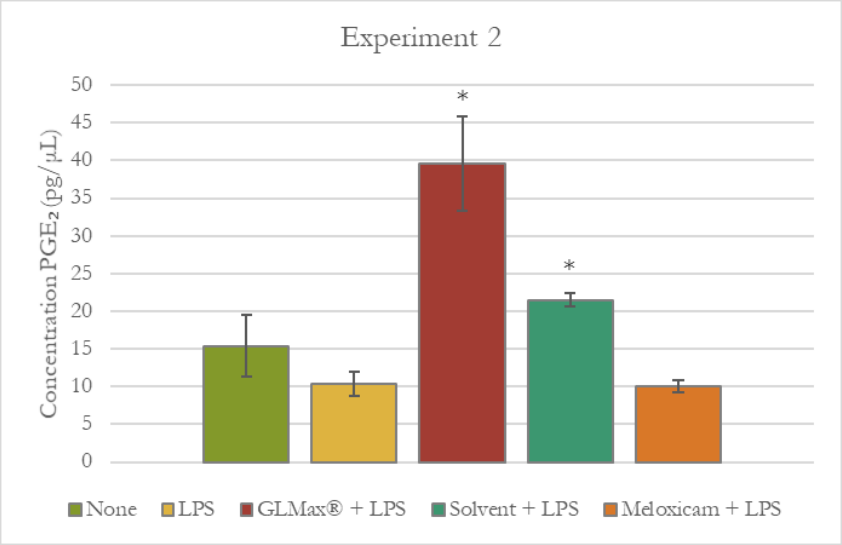
The effect of 0.1 mg/ml meloxicam on PGE_2 production combined with LPS challenge was also measured. In the first and third experiments, adding meloxicam significantly reduced PGE_2 levels, in the second experiment no significant effect was observed. Measurements from the first experiment showed a 31,13% decrease in PGE_2. In the last experiment, a 45,37% decrease was observed.

4.2.1.3 Effect of solvent

In the first and third experiments, the solvent solution did not have any effect on PGE_2 levels compared to untreated cells challenged with LPS. In the second experiment, the cells produced 207,95% more PGE_2.

Figure 6: PGE_2 levels produced by equine PBMCs, after pre-incubation with GLMax®, Solvent or Meloxicam, and incubation with the same components and LPS.





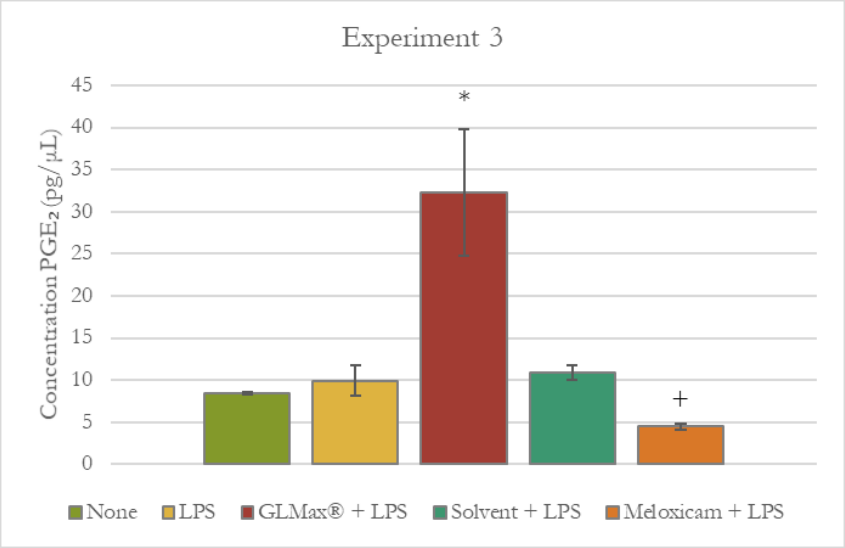


Figure 6: **PGE_2 levels produced by equine PBMCs, after pre-incubation with GLMax®, Solvent or Meloxicam and incubation with the same components and LPS.** PGE_2 was measured with HPLC in supernatants collected after the cell culture experiments. Bars display mean PGE_2 levels of three replicates. Error bars display +/- 1 standard deviation. * displays a significantly higher level of PGE_2 compared to PBMCs that were only stimulated with LPS. + significantly lower level of PGE_2 compared to PBMCs that were only stimulated with LPS.

4.3 Cytotoxicity of GLMax®

The cytotoxicity of the test compounds was studied in the second and third experiments. Results from the viability assays can be found in figure 7. Both GLMax® and the solvent increased viability of equine PBMCs, but significant effects could not be demonstrated due to the high variance in the results, causing a significant result in Levene's test of homogeneity. Meloxicam significantly increased viability at a concentration of 0,01 mg/ml and 0,1 mg/ml in the first experiment. In the second experiment, viability was significantly increased after treatment with 0,1 mg/ml meloxicam.

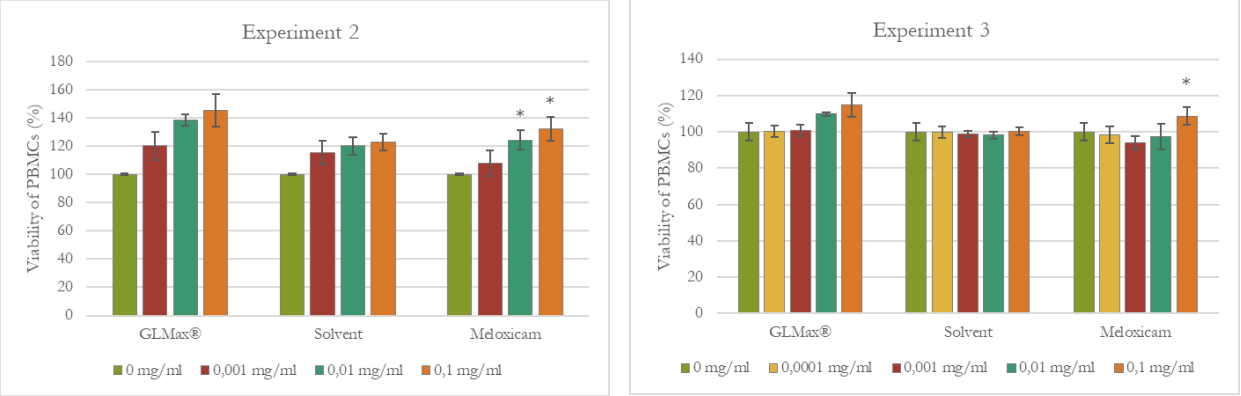


Figure 7: **Viability of equine PBMCs, after incubation with GLMax®, Solvent or Meloxicam and Alamarblue™.** The cytotoxicity assay was performed by incubating equine PBMCs with the test compounds and Alamarblue™. The bars display the mean viability of five replicates. Error bars display +/- 1 standard deviation.

5. Conclusion and discussion

5.1 Influence of GLMax® on inflammatory mediator production

In this study, we found that GLMax® seems to influence TNF-α levels, however, only in a moderate way and it was difficult to reproduce, even in the same study. As discussed in the Results section, in the first and second experiments it was found GLMax® inhibited TNF-α production in some cases compared to the negative control, but this effect could not be repeated in the third experiment. Moreover, only a concentration of 0,001 mg/ml was found to significantly inhibit TNF-α levels after pre-incubation and incubation with GLMax® in two experiments. None of the significant effects that were found after only pre-incubation with the compound could be repeated. The patterns of inhibition that were found during the experiments could not be fitted to a dose-response curve. Instead of a dose-response curve (either increasing or decreasing) the line following different concentrations showed an irregular pattern.

In the introduction, it was already discussed that there is evidence GLM decreases TNF-α excretion. Two *in vitro* studies found a decrease of TNF-α excretion when several cell lines were treated with Perna®.[5,10] In two other studies GLM was fed to rats with experimentally induced arthritis. In this study, researchers found that splenocytes extracted from these rats produced less TNF-α.[4,13] In this study, we were not able to confirm this effect, sometimes some effects where observed, but reproducing these effects in additional experiments was not possible.

0.1 mg/ml GLMax® did not decrease PGE_2 production, but significantly increased PGE_2 levels in all experiments. There was no significant difference between cells incubated in blank medium and cells incubated with LPS. This result is remarkable since in two previous studies[10,12] researchers found that GLM showed moderate to strong inhibition of COX-1 and COX-2. In another study, it was found that GLM inhibits PGE_2 production in LPS-challenged human monocytes.[9] We were not able to reproduce these results but found the opposite. It seems likely equine PBMCs react to a compound in GLMax®.

5.1.1 Influence of GLMax® compared to solvent:

The solvent solution was found to inhibit TNF-α production several times, but also significantly increase TNF-α levels in other cases. The lowest concentration (0,001 mg/ml) increased TNF-α levels in the first experiment, both after pre-incubation and pre-incubation + incubation, but decreased TNF-α levels in the second experiment. In the second experiment, the solvent effectively and significantly lowered TNF-α levels at all concentrations. Compared to GLMax® it was more effective in the second experiment. Especially the higher concentrations of solvent were more effective in the first and second experiments than the same concentrations of GLMax® in the same experiments. In the third experiment, neither of the substances was effective, the solvent solution at the lowest concentration even increased TNF-α levels. The solvent solution at 0.1 mg/ml produced no significant effect on PGE_2 production in any of the experiments, measured levels were comparable to those measured when cells were incubated with LPS or blank medium.

It was not expected that the solvent would have anti-inflammatory properties (at least concerning TNF-α production), since it's a relatively simple mixture not containing substances that have anti-inflammatory bioactive properties *in vivo*. Especially the fact that it was generally more effective than GLMax® was very unexpected. The fact that the solvent mixture was effective in some cases might be an indication that the differences in TNF-α levels are in fact not caused by the adding of the test compounds, but have some other reason. The fact that the solvent solution sometimes increases and in other cases decreases TNF-α levels supports this as well. This is further supported by the fact that in the case of the solvent solution, we were also unable to fit a dose-response curve to the obtained data.

5.1.2 Influence of GLMax® compared to meloxicam:

Meloxicam produced slight, but significant decreases in TNF-α levels, but in other cases increased TNF-α levels. It was not necessarily expected meloxicam would reduce TNF-α levels. Meloxicam increasing TNF-α levels was also unexpected, and could be due to contamination. There are reports of meloxicam effectivity after LPS stimulation in equine whole blood assays[41,42], but in neither of the studies, TNF-α levels were investigated. In the first experiment we did, 0,1 mg/ml meloxicam significantly increased TNF-α, but in the second experiment, the same concentration significantly reduced TNF-α. The variation in these results cannot be explained by the reasons discussed above for the solvent and GLMax® mixtures.

As was to be expected, Meloxicam significantly reduced PGE_2 levels in the first and third experiments, however, this effect was not visible in the second experiment. Since Meloxicam is a COX inhibitor, it is not surprising it would decrease PGE_2 production. Indeed, Beretta et al[41] found a strong inhibition of PGE_2 production by meloxicam in an LPS-challenged equine whole blood assay. The fact that Meloxicam did not decrease PGE_2 levels, although it is a well-known COX-inhibitor, makes it even more plausible there was a contamination present during the experiment. Altogether, the results from the second experiment are possibly invalid, because it is very unexpected Meloxicam was not able to decrease PGE_2 levels.

5.1.3 Factors influencing results:

In general, it can be said the experiments produced varying results, where all three test compounds seemed to increase TNF-α in some cases and decrease TNF-α in other cases. In the case of PGE_2, the results are more consistent, however, not in favor of GLMax® effectivity nor the validity of the experiment. There are several possible reasons why the results were inconsistent for TNF- α and negative for PGE_2. Since GLMax® is a neutraceutical, directly produced from the green-lipped mussel, it is a very complex mixture. As discussed in the introduction, it is made up of a long list of biochemical components, of which several are suspected to be bioactive. When these components are used in *in vitro* research, it is extremely difficult to predict what substances and which concentrations are present in the experiments. Moreover, the composition of GLMax® is fluid, but it contained components that were only moderately solvable. This irregularity can be seen in the variance of the first TNF- α measurements, where the variance of the GLMax® and solvent measurements is very high, but the variance of meloxicam is much lower. The same can be said for the second and third PGE_2 measurements, where variance in the GLM group was much higher compared to the variance in the meloxicam group. Since the meloxicam used in this experiment is only one component, and GLMax® contains a variety of molecules, differences in variance are not surprising. This also makes it much harder to predict and prove what the exact effect of GLMax® is *in vitro*.

It is however unlikely that GLMax® contains a substance that binds LPS, and in that way caused a decrease in TNF-α levels. If this would have been the case, we would have seen no activity when PBMCs were pre-incubated with GLMax® but would have seen some consistent TNF-α decrease when PBMCs were incubated with GLMax® and LPS at the same time. In our study, we found varying activity after pre-incubation and preincubation + incubation. It could however be that some variation in the TNF-α is caused by the difference in reactivity of equine PBMCs to LPS. It has been reported previously that equine PBMCs originating from different donors show different reactivity.[8] This could be at least an explanation for some variety in the results. However, it would be expected that if this was the only reason, all experiments would show more or less the same pattern, and differences would mostly be found in the strength of the effect.

Another reason why there might be some variation in results is that there is possibly some contamination of GLMax® and the solvent solution with components stimulating inflammatory mediator production. As discussed in the results section, in all three experiments incubation with 0,1 mg/ml GLMax® significantly increased TNF-α levels in the absence of LPS. The observed increase in TNF-α after incubation with GLMax® in the first and third experiments was 166,2179 and 165,0382 pg/ml. These levels are about the same as the observed TNF-α levels after stimulation with 0,1 ng/ml LPS. In the second experiment, the TNF-α level after incubation with 0,1 mg/ml GLMax® was even higher, 682,2569 pg/ml. Especially the fact that PGE_2 levels increase significantly in the presence of GLMax® + LPS, between 325% and 382%, but not in the presence of only LPS, makes it likely that there is some form of contaminant or compound in GLMax® which stimulates inflammatory mediator production. In the case of the solvent solution, high levels of TNF-α were found, but there was no significant effect on PGE_2. It seems likely that there is some contamination in GLMax® and possibly in the solvent, causing effects opposite to what was expected. This could partly explain the variation in results; it might be possible GLMax® contains compounds stimulating TNF-α and PGE_2 production as well as compounds inhibiting TNF-α production. Depending on the concentration of these compounds, different effects are observed in the experiments.

It is also possible some compound in GLMax® causes desensitization of PBMCs for LPS, which has been previously suggested[8] but does not decrease TNF-α levels by actively inhibiting production. However, the concentration of TNF-α reaches a plateau when PBMCs are stimulated with LPS at high concentrations. The LPS concentration used in this study was high enough to be in the plateau (see results section), but TNF-α levels were still higher than the controls at some point. This would imply that GLMax® is not contaminated with LPS, but with some other substance causing increasing TNF-α production through another pathway.

Taken in to account all of the reasons discussed above, it seems likely that the variation in these experiments is not caused by a single reaction, but that several processes are going on at once. The high variance in many measurements, the fact that we were unable to fit a dose-response curve to the results, the difficulty in replicating our experiments, the increase in TNF-α in the absence of LPS, the increase in PGE_2, and the complexity of the GLMax® mixture all support this.

5.2 Recommendations for future research

Previous research as discussed in the introduction seems to produce moderately promising results, however, we were not able to confirm this in our study. However, as discussed above, GLMax® is a complex mixture, consisting of many different compounds. Especially combined with equine PBMCs that vary in reactivity depending on the donor, this produces results with a high variance. For *in vitro* research, it would be advisable to analyze all components (as far as possible), separate them and evaluate every possibly bioactive component individually. This is however a daunting and difficult task and would be rather time-consuming to realize. Moreover, equine PBMCs have their limitations, such as a short *in vitro* lifespan and the aforementioned difference in reaction strength. Results with lower variance could probably be obtained by the use of cell lines, but equine cell lines are unfortunately not available to date.

The complex mixture also makes it difficult to predict how *in vitro* results could be translated into *in vivo* efficacy. Instead of researching every GLMax® component separately, it seems more efficient to do an *in vivo* study in horses. Effects on TNF- α and PGE_2 production could rather easily be measured in blood samples, maybe even in synovial fluid. By comparing equine patients treated with meloxicam, and horses treated with meloxicam and GLMax®, it can be avoided to leave patients possibly untreated by giving them a possibly ineffective neutraceutical. At the same time, not only biochemical effects but also clinical effects such as lameness and pain scores can be evaluated.

5.3 Conclusion

In conclusion, it can be said that GLMax® does not exhibit consistent anti-inflammatory effects in this study. We were unable to reproduce results from previous research in other models and other species (using other GLM extracts). There are probably too many factors influencing inflammatory mediator production *in vitro* in this setup, complicating result analyses. In the case of TNF-α, results have high variance and vary between experiments. PGE_2 levels even increased when cells were incubated with GLMax®. The results show that GLMax® is at best ineffective as an anti-inflammatory compound *in vitro* and possibly even has inflammatory properties *in vitro*.

6. Literature

1. Wakimoto T, Kondo H, Nii H, et al. Furan fatty acid as an anti-inflammatory component from the green-lipped mussel perna canaliculus. *Proc Natl Acad Sci U S A*. 2011;108(42):17533-17537. doi: 10.1073/pnas.1110577108 [doi].

2. Grienke U, Silke J, Tasdemir D. Bioactive compounds from marine mussels and their effects on human health. *Food Chemistry*. 2014;142:48-60. doi: https://doi.org/10.1016/j.foodchem.2013.07.027.

3. Coulson S, Butt H, Vecchio P, Gramotnev H, Vitetta L. Green-lipped mussel extract (*Perna canaliculus*) and glucosamine sulphate in patients with knee osteoarthritis: Therapeutic efficacy and effects on gastrointestinal microbiota profiles. *Inflammopharmacology*. 2013;21(1):79-90.

4. Lee C, Butt YK, Wong M, Lo SC. A lipid extract of perna canaliculus affects the expression of pro-inflammatory cytokines in a rat adjuvant-induced arthritis model. *European annals of allergy and clinical immunology*. 2008;40(4):148.

5. Lawson BR, Belkowski SM, Whitesides JF, Davis P, Lawson JW. Immunomodulation of murine collagen-induced arthritis by N, N-dimethylglycine and a preparation of perna canaliculus. *BMC complementary and alternative medicine*. 2007;7(1):20.

6. Buddhachat K, Siengdee P, Chomdej S, Soontornvipart K, Nganvongpanit K. Effects of different omega-3 sources, fish oil, krill oil, and green-lipped mussel against cytokine-mediated canine cartilage degradation. *In Vitro Cellular & Developmental Biology-Animal*. 2017;53(5):448-457.

7. Vendrig JC, Coffeng L, Fink-Gremmels J. Equine colostral carbohydrates reduce lipopolysaccharide-induced inflammatory responses in equine peripheral blood mononuclear cells. *Equine Vet J*. 2012;44(S43):68-72.

8. Vendrig JC, Coffeng LE, Fink-Gremmels J. In vitro evaluation of defined oligosaccharide fractions in an equine model of inflammation. *BMC veterinary research*. 2013;9(1):147.

9. Whitehouse M, Macrides T, Kalafatis N, Betts W, Haynes D, Broadbent J. Anti-inflammatory activity of a lipid fraction (lyprinol) from the NZ green-lipped mussel. *Inflammopharmacology*. 1997;5(3):237-246.

10. Mani S, Lawson JW. In vitro modulation of inflammatory cytokine and IgG levels by extracts of perna canaliculus. *BMC complementary and alternative medicine*. 2006;6(1):1.

11. Pearson W, Orth MW, Karrow NA, MacLusky NJ, Lindinger MI. Anti-inflammatory and chondroprotective effects of nutraceuticals from sasha's blend in a cartilage explant model of inflammation. *Molecular nutrition & food research*. 2007;51(8):1020-1030.

12. McPhee S, Hodges L, Wright P, et al. Anti-cyclooxygenase effects of lipid extracts from the new zealand green-lipped mussel, perna canaliculus. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 2007;146(3):346-356.

13. Lee CH, Lum JH, Ng CK, et al. Pain controlling and cytokine-regulating effects of lyprinol, a lipid extract of perna canaliculus, in a rat adjuvant-induced arthritis model. *Evid Based Complement Alternat Med*. 2009;6(2):239-245. doi: 10.1093/ecam/nem100 [doi].

14. Lee C, Butt Y, Wong M, Lo S. Differential protein expression induced by a lipid extract of perna canaliculus in splenocytes of rats with adjuvant-induced arthritis. *Inflammopharmacology*. 2008;16(4):188-194.

15. Singh M, Hodges L, Wright P, et al. The CO2-SFE crude lipid extract and the free fatty acid extract from perna canaliculus have anti-inflammatory effects on adjuvant-induced arthritis in rats. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 2008;149(2):251-258.

16. Torres DM, Tooley KL, Butler RN, Smith CL, Geier MS, Howarth GS. Lyprinol™ only partially improves indicators of small intestinal integrity in a rat model of 5-fluorouracil-induced mucositis. *Cancer biology & therapy*. 2008;7(2):295-302.

17. Tenikoff D, Murphy KJ, Le M, Howe PR, Howarth GS. Lyprinol (stabilised lipid extract of new zealand green-lipped mussel): A potential preventative treatment modality for inflammatory bowel disease. *J Gastroenterol*. 2005;40(4):361-365.

18. Bui LM, Bierer TL. Influence of green lipped mussels (perna canaliculus) in alleviating signs of arthritis in dogs. *Veterinary Therapeutics*. 2003;4(4):397-407.

19. Bierer TL, Bui LM. Improvement of arthritic signs in dogs fed green-lipped mussel (perna canaliculus). *J Nutr*. 2002;132(6 Suppl 2):1634S-6S.

20. Pollard B, Guilford W, Ankenbauer-Perkins K, Hedderley D. Clinical efficacy and tolerance of an extract of green-lipped mussel (perna canaliculus) in dogs presumptively diagnosed with degenerative joint disease. *N Z Vet J*. 2006;54(3):114-118.

21. Kwananocha I, Vijarnsorn M, Kashemsant N, Lekcharoensuk C. Effectiveness of disease modifying osteoarthritis agents and carprofen for treatment of canine osteoarthritis. *The Thai Journal of Veterinary Medicine*. 2016;46(3):363.

22. Mongkon N, Soontornvipart K. Preliminary study of the clinical outcome of using PCSO-524 polyunsaturated fatty acid compound in the treatment of canine osteoarthritis and degenerative spinal diseases. *The Thai Journal of Veterinary Medicine*. 2012;42(3):311.

23. Soontornvipart K, Mongkhon N, Nganvongpanit K, Kongtawelert P. Effect of PCSO-524 on OA biomarkers and weight-bearing properties in canine shoulder and coxofemeral osteoarthritis. *The Thai Journal of Veterinary Medicine*. 2015;45(2):157-165.

24. Cayzer J, Hedderley D, Gray S. A randomised, double-blinded, placebo-controlled study on the efficacy of a unique extract of green-lipped mussel (perna canaliculus) in horses with chronic fetlock lameness attributed to osteoarthritis. *Equine Vet J*. 2012;44(4):393-398.

25. Dobenecker B, Beetz Y, Kienzle E. A placebo-controlled double-blind study on the effect of nutraceuticals (chondroitin sulfate and mussel extract) in dogs with joint diseases as perceived by their owners. *J Nutr*. 2002;132(6 Suppl 2):1690S-1S.

26. Emelyanov A, Fedoseev G, Krasnoschekova O, Abulimity A, Trendeleva T, Barnes PJ. Treatment of asthma with lipid extract of new zealand green-lipped mussel: A randomised clinical trial. *Eur Respir J*. 2002;20(3):596-600.

27. Gruenwald J, Graubaum H, Hansen K, Grube B. Efficacy and tolerability of a combination of lyprinol® and high concentrations of EPA and DHA in inflammatory rheumatoid disorders. *Adv Ther*. 2004;21(3):197-201.

28. Coulson S, Palacios T, Vitetta L. Perna canaliculus (green-lipped mussel): Bioactive components and therapeutic evaluation for chronic health conditions. In: *Novel natural products: Therapeutic effects in pain, arthritis and gastro-intestinal diseases*. Springer; 2015:91-132.

29. Bui LM, Bierer TL. Influence of green lipped mussels (perna canaliculus) in alleviating signs of arthritis in dogs. *Veterinary Therapeutics*. 2003;4(4):397-407.

30. Murphy K, Mann N, Sinclair A. Fatty acid and sterol composition of frozen and freeze-dried new zealand green lipped mussel (perna canaliculus) from three sites in new zealand. *Asia Pac J Clin Nutr*. 2003;12(1):50-60.

31. Qu J, Mélot C, Appelboom T. Synofit premium in refractory low back pain: A retrospective observational study. *Open Journal of Rheumatology and Autoimmune Diseases*. 2017;7(02):120.

32. Treschow A, Hodges L, Wright P, Wynne P, Kalafatis N, Macrides T. Novel anti-inflammatory ω-3 PUFAs from the new zealand green-lipped mussel, perna canaliculus. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 2007;147(4):645-656.

33. Juliano C, Manconi P, Cossu M. Characterisation of commercial perna canaliculus samples and development of extemporaneous oral veterinary paste formulations containing perna. *Natural product research*. 2016;30(18):2041-2048.

34. Scotti PD, Dearing SC, Greenwood DR, Newcomb RD. Pernin: A novel, self-aggregating haemolymph protein from the new zealand green-lipped mussel, perna canaliculus (bivalvia: Mytilidae). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 2001;128(4):767-779. doi: https://doi.org/10.1016/S1096-4959(01)00301-3.

35. Miller T, Dodd J, Ormrod D, Geddes R. Anti-inflammatory activity of glycogen extracted fromPerna canaliculus (NZ green-lipped mussel). *Agents Actions*. 1993;38(2):C139-C142.

36. Ahmad TB, Liu L, Kotiw M, Benkendorff K. Review of anti-inflammatory, immune-modulatory and wound healing properties of molluscs. *J Ethnopharmacol*. 2018;210:156-178.

37. Wood L, Hazlewood L, Foster P, Hansbro P. Lyprinol reduces inflammation and improves lung function in a mouse model of allergic airways disease. *Clinical & Experimental Allergy*. 2010;40(12):1785-1793.

38. Whitehouse M, Butters D. Combination anti-inflammatory therapy: Synergism in rats of NSAIDs/corticosteroids with some herbal/animal products. *Inflammopharmacology*. 2003;11(4-6):453-464.

39. Grauw Jd, Lest Cv, Brama P, Rambags B, Weeren PRv. In vivo effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis. *Equine Vet J*. 2009;41(7):693-699.

40. Banse H, Cribb AE. Comparative efficacy of oral meloxicam and phenylbutazone in 2 experimental pain models in the horse. *Can Vet J*. 2017;58(2):157-167.

41. Beretta C, Garavaglia G, Cavalli M. COX-1 and COX-2 inhibition in horse blood by phenylbutazone, flunixin, carprofen and meloxicam: An in vitro analysis. *Pharmacological research*. 2005;52(4):302-306.

42. Brideau C, Van Staden C, Chan CC. In vitro effects of cyclooxygenase inhibitors in whole blood of horses, dogs, and cats. *Am J Vet Res*. 2001;62(11):1755-1760.

Appendix A: Cell culture experiment 1 and 2 plate setup

For all plates:
GLM = green lipped mussel dilution
MEL = meloxicam dilution
C1 = concentration 1 = 0.1 mg/ml
C2 = concentration 2 = 0.01 mg/ml
C3 = concentration 3 = 0.001 mg/ml
0 = blank medium
+LPS = challenged with LPS

Pre-incubation:

Plate 1 and 2:

	1	**2**	**3**	**4**	**5**	**6**
A	0	0	0	0	0	0
B	C1 GLM	C1 GLM	C1 GLM	C1 GLM	C1 GLM	C1 GLM
C	C2 GLM	C2 GLM	C2 GLM	C2 GLM	C2 GLM	C2 GLM
D	C3 GLM	C3 GLM	C3 GLM	C3 GLM	C3 GLM	C3 GLM

Plate 3 and 4:

	1	**2**	**3**	**4**	**5**	**6**
A	0	0	0	0	0	0
B	C1 Solvent	C1 Solvent	C1 Solvent	C1 Solvent	C1 Solvent	C1 Solvent
C	C2 Solvent	C2 Solvent	C2 Solvent	C2 Solvent	C2 Solvent	C2 Solvent
D	C3 Solvent	C3 Solvent	C3 Solvent	C3 Solvent	C3 Solvent	C3 Solvent

Plate 5 and 6:

	1	2	3	4	5	6
A	0	0	0	0	0	0
B	C1 MEL	C1 MEL	C1 MEL	C1 MEL	C1 MEL	C1 MEL
C	C2 MEL	C2 MEL	C2 MEL	C2 MEL	C2 MEL	C2 MEL
D	C3 MEL	C3 MEL	C3 MEL	C3 MEL	C3 MEL	C3 MEL

Incubation:

Plate 1:

	1	2	3	4	5	6
A	+LPS 0	+LPS 0	+LPS 0	0	0	0
B	+LPS 0	+LPS 0	+LPS 0	0	0	0
C	+LPS 0	+LPS 0	+LPS 0	0	0	0
D	+LPS 0	+LPS 0	+LPS 0	0	0	0

Plate 2:

	1	2	3	4	5	6
A	+LPS 0	+LPS 0	+LPS 0	0	0	0
B	+LPS C1 GLM	+LPS C1 GLM	+LPS C1 GLM	C1 GLM	C1 GLM	C1 GLM
C	+LPS C2 GLM	+LPS C2 GLM	+LPS C2 GLM	C2 GLM	C2 GLM	C2 GLM
D	+LPS C3 GLM	+LPS C3 GLM	+LPS C3 GLM	C3 GLM	C3 GLM	C3 GLM

Plate 3:

	1	2	3	4	5	6
A	+LPS 0	+LPS 0	+LPS 0	0	0	0
B	+LPS 0	+LPS 0	+LPS 0	0	0	0
C	+LPS 0	+LPS 0	+LPS 0	0	0	0
D	+LPS 0	+LPS 0	+LPS 0	0	0	0

Plate 4:

	1	2	3	4	5	6
A	+LPS 0	+LPS 0	+LPS 0	0	0	0
B	+LPS C1 Solvent	+LPS C1 Solvent	+LPS C1 Solvent	C1 Solvent	C1 Solvent	C1 Solvent
C	+LPS C2 Solvent	+LPS C2 Solvent	+LPS C2 Solvent	C2 Solvent	C2 Solvent	C2 Solvent
D	+LPS C3 Solvent	+LPS C3 Solvent	+LPS C3 Solvent	C3 Solvent	C3 Solvent	C3 Solvent

Plate 5:

	1	2	3	4	5	6
A	+LPS 0	+LPS 0	+LPS 0	0	0	0
B	+LPS 0	+LPS 0	+LPS 0	0	0	0
C	+LPS 0	+LPS 0	+LPS 0	0	0	0
D	+LPS 0	+LPS 0	+LPS 0	0	0	0

Plate 6:

	1	2	3	4	5	6
A	+LPS 0	+LPS 0	+LPS 0	0	0	0
B	+LPS C1 MEL	+LPS C1 MEL	+LPS C1 MEL	C1 MEL	C1 MEL	C1 MEL
C	+LPS C2 MEL	+LPS C2 MEL	+LPS C2 MEL	C2 MEL	C2 MEL	C2 MEL
D	+LPS C3 MEL	+LPS C3 MEL	+LPS C3 MEL	C3 MEL	C3 MEL	C3 MEL

Appendix B: Cell culture experiment 3 plate setup

For all plates:
GLM = green lipped mussel dilution
MEL = meloxicam dilution
C1 = concentration 1 = 0.1 mg/ml
C2 = concentration 2 = 0.01 mg/ml
C3 = concentration 3 = 0.001 mg/ml
C4 = concentration 4 = 0.0001 mg/ml
0 = blank medium
+LPS = challenged with LPS

Pre-incubation:

Plate 1 and 2:

	1	**2**	**3**	**4**	**5**	**6**
A	C1 GLM	C1 GLM	C1 GLM	C1 GLM	C1 GLM	C1 GLM
B	C2 GLM	C2 GLM	C2 GLM	C2 GLM	C2 GLM	C2 GLM
C	C3 GLM	C3 GLM	C3 GLM	C3 GLM	C3 GLM	C3 GLM
D	C4 GLM	C4 GLM	C4 GLM	C4 GLM	C4 GLM	C4 GLM

Plate 4 and 5:

	1	**2**	**3**	**4**	**5**	**6**
A	C1 Solvent	C1 Solvent	C1 Solvent	C1 Solvent	C1 Solvent	C1 Solvent
B	C2 Solvent	C2 Solvent	C2 Solvent	C2 Solvent	C2 Solvent	C2 Solvent
C	C3 Solvent	C3 Solvent	C3 Solvent	C3 Solvent	C3 Solvent	C3 Solvent
D	C4 Solvent	C4 Solvent	C4 Solvent	C4 Solvent	C4 Solvent	C4 Solvent

Plate 7 and 8:

	1	2	3	4	5	6
A	C1 MEL	C1 MEL	C1 MEL	C1 MEL	C1 MEL	C1 MEL
B	C2 MEL	C2 MEL	C2 MEL	C2 MEL	C2 MEL	C2 MEL
C	C3 MEL	C3 MEL	C3 MEL	C3 MEL	C3 MEL	C3 MEL
D	C4 MEL	C4 MEL	C4 MEL	C4 MEL	C4 MEL	C4 MEL

Plate 3, 6 and 9:

	1	2	3	4	5	6
A	0	0	0	0	0	0
B	0	0	0	0	0	0
C	0	0	0	0	0	0
D	0	0	0	0	0	0

Incubation:

Plate 1:

	1	2	3	4	5	6
A	+LPS C1 GLM	+LPS C1 GLM	+LPS C1 GLM	+LPS 0	+LPS 0	+LPS 0
B	+LPS C2 GLM	+LPS C2 GLM	+LPS C2 GLM	+LPS 0	+LPS 0	+LPS 0
C	+LPS C3 GLM	+LPS C3 GLM	+LPS C3 GLM	+LPS 0	+LPS 0	+LPS 0
D	+LPS C4 GLM	+LPS C4 GLM	+LPS C4 GLM	+LPS 0	+LPS 0	+LPS 0

Plate 2:

	1	2	3	4	5	6
A	C1 GLM	C1 GLM	C1 GLM	0	0	0
B	C2 GLM	C2 GLM	C2 GLM	0	0	0
C	C3 GLM	C3 GLM	C3 GLM	0	0	0
D	C4 GLM	C4 GLM	C4 GLM	0	0	0

Plate 4:

	1	2	3	4	5	6
A	+LPS C1 Solvent	+LPS C1 Solvent	+LPS C1 Solvent	+LPS 0	+LPS 0	+LPS 0
B	+LPS C2 Solvent	+LPS C2 Solvent	+LPS C2 Solvent	+LPS 0	+LPS 0	+LPS 0
C	+LPS C3 Solvent	+LPS C3 Solvent	+LPS C3 Solvent	+LPS 0	+LPS 0	+LPS 0
D	+LPS C4 Solvent	+LPS C4 Solvent	+LPS C4 Solvent	+LPS 0	+LPS 0	+LPS 0

Plate 5:

	1	2	3	4	5	6
A	C1 Solvent	C1 Solvent	C1 Solvent	0	0	0
B	C2 Solvent	C2 Solvent	C2 Solvent	0	0	0
C	C3 Solvent	C3 Solvent	C3 Solvent	0	0	0
D	C4 Solvent	C4 Solvent	C4 Solvent	0	0	0

Plate 7:

	1	2	3	4	5	6
A	C1 MEL	C1 MEL	C1 MEL	+LPS 0	+LPS 0	+LPS 0
B	C2 MEL	C2 MEL	C2 MEL	+LPS 0	+LPS 0	+LPS 0
C	C3 MEL	C3 MEL	C3 MEL	+LPS 0	+LPS 0	+LPS 0
D	C4 MEL	C4 MEL	C4 MEL	+LPS 0	+LPS 0	+LPS 0

Plate 8:

	1	2	3	4	5	6
A	+LPS C1 MEL	+LPS C1 MEL	+LPS C1 MEL	0	0	0
B	+LPS C2 MEL	+LPS C2 MEL	+LPS C2 MEL	0	0	0
C	+LPS C3 MEL	+LPS C3 MEL	+LPS C3 MEL	0	0	0
D	+LPS C4 MEL	+LPS C4 MEL	+LPS C4 MEL	0	0	0

Plate 3, 6 and 9:

	1	2	3	4	5	6
A	0	0	0	0	0	0
B	+LPS 0	+LPS 0	+LPS 0	+LPS 0	+LPS 0	+LPS 0
C	10 ng/ml LPS	10 ng/ml LPS	10 ng/ml LPS	1 ng/ml LPS	1 ng/ml LPS	1 ng/ml LPS
D	0.1 ng/ml LPS	0.1 ng/ml LPS	0.1 ng/ml LPS	0.01 ng/ml LPS	0.01 ng/ml LPS	0.01 ng/ml LPS