Short Communication

Dietary Supplementation of Dodecanoic Acid in Maternal Mice Enhances Resistance to *Giardia duodenalis* Infection in Suckling Neonatal Pups

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ABSTRACT: Background: Studies have confirmed that dodecanoic acid (DA; C: 12; common name: lauric acid) has anti-giardial properties against *G. duodenalis* trophozoites *in vitro* with an LD_{50} concentration comparable to that of metronidazole, the drug of choice in the treatment of giardiasis. DA appears to induce trophozoite death by accumulating within the parasite cytoplasm resulting in rupture of the trophozoite cell membrane. In this study, the dietary supplementation of dodecanoic acid (and other compounds containing DA) to maternal mice was examined to determine whether such dietary changes could enhance resistance to *G.duodenalis* infection in suckling neonatal pups. **Materials and Methods:** Each maternal mouse was offered a dietary supplement of either DA (mean intake of 0.81 and 0.3 gm/day), coconut oil (mean intake of 0.86 gm of dodecanoic acid/day) or Monolaurin™(mean intake of 1.18 gm/day), whereas the negative control maternal mice received normal feed pellets 20 gm/day containing no DA or DA derivatives (no supplementation) for 8 days (3 days ante-partum and then 5 days post-partum).At day 3 of age, all pups from the litters were then orogastrically inoculated with 1×10^5 *G. duodenalis* trophozoites. On day 6 post-inoculation, each pup was euthanized and the entire intestinal tract from the pyloric sphincter to the rectum was surgically removed from each necropsied animal. The mean counts of total parasite burdens [trophozoite and cyst] were calculated on the gut washes of each animal. **Results**: In Trial 1, the pups of the maternal mouse that received a mean intake of 0.81 g ± 0.58 per day of dodecanoic acid were clear of any *G. duodenalis* trophozoites or cysts. In contrast, the control pups had a mean total parasite burden of 6.2 ± 7.6 x 10^5. In Trial 2, the pups of the maternal mouse that received a mean intake of 0.30 ± 0.05 g per day of dodecanoic acid were also clear of any *G. duodenalis* trophozoites or cysts, however the control pups had a mean total parasite burden of 8.6 ± 3.5 x 10^5. In Trial 3, the pups of the maternal mouse that received a mean intake of 1.72 g ± 0.42 per coconut oil [which gave a calculated average daily intake of 0.86 ± 0.21 g of dodecanoic acid] demonstrated a mean total parasite burden of 5.1 ± 6.3 x 10^5, (both trophozoites and cysts), whereas the control pups had a mean total parasite burden of 18.2 ± 11.07 x 10^5. In Trial 4, all pups of the maternal mouse that received a daily mean intake of 1.18g ± 0.32 per day of Monolaurin (dodecanoic monoglyceride) had a mean total of 7.0 ± 4.03 x 10^5 parasite burden (both trophozoites and cysts) which was similar to the control pups that possessed a mean total parasite burden of 7.75 ± 6.36 x 10^5. **Conclusion**: These studies have opened fresh avenues for development of natural drug therapy in which food supplementation may augment, or even replace, some of the standard chemotherapeutic agents presently employed in the treatme of giardiasis and possibly other infectious protozoal intestinal diseases.

KEYWORDS: Dodecanoic Acid, *Giardia duodenalis* and *in vivo* trials, metronidazole, coconut oil, lauric acid.

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INTRODUCTION

To understand the importance of animal-based research, one must realize the impact it has had, and continues to have, on medical research, scientific discovery and human health-care. Animal models are used because of their comparative similarities with the physiology of humans and to gain a clearer understanding of mamma-

lian anatomical function. Since humans and some other mammals share a number of common diseases, animals can act as experimental models to study such diseases. Data obtained from animal studies are essential before new therapeutic techniques, drug candidates, and surgical procedures can be offered in human health-care. Identification and legal administration of new drugs usually require animal testing as medical researchers and drug companies must be able to ascertain a compound's effects, both beneficial and harmful, on the full range of organs and tissues of the mammalian body. Extensive analyses and documentation of the efficacies and safety of new drugs, treatment regimes and modes of administration are standard requirements before approval for human clinical trials.[1,2]

Roberts-Thomson *et al.* reported the successful infection of mice with *Giardia muris*, and the subsequent transfer of this infection to other mice. Since then, clinical investigators have attempted to infect rats, mice, dogs and other mammals with trophozoites of *G. duodenalis* in order to explore the transmission of infection and pathogenesis of mammalian giardiasis. Hill *et al.*[3] also demonstrated the ability of *G. duodenalis* trophozoites to infect and complete their life cycle in neonatal mice. Their studies found that infection occurred in all pups inoculated at three, seven, and 14 days of age, but not in mice older than 21 days[2–3].

The neonatal murine model has since offered researchers a valuable experimental tool to explore the many parameters of the pathogenesis of *G. duodenalis* infection, including intestinal villus atrophy, crypt hyperplasia, crypt cell proliferation rate, decreased mucosal disaccharidases, and increased intraepithelial lymphocyte numbers within the intestinal mucosa of the infected host.[1,5–14] Additionally, investigators have noted that after exposure to *Giardia* spp. the immunity of some mammals to the parasite can be passively transferred to their young through the maternal breast milk. There are now numerous reports confirming the beneficial effects of maternal breast milk against giardiasis.[15–19]

Mammalian breast milk contains a range of fats, proteins, carbohydrates and minerals. The main fats in mammalian milk are the fatty acids decanoic (C10), dodecanoic (C12), octanoic (C18), hexadecanoic (C16) and linoleic (C18) acids.[20,21] Research suggests that these fatty acids are taken up through the diet and transported throughout the body via the blood and lymphatic circulatory systems. A proportion of these fatty acids are then expressed in maternal breast milk of lactating mammals, including humans. The fatty acids appear to be key components in protect-

ing a neonate's intestinal tract from bacterial, viral, fungal and protozoal infections until the immature immune system reaches competency.[16] The fatty acid profile of the maternal milk can be altered by diet, and supplementation of the diet with a specific fatty acid can subsequently increase the expression of that particular fatty acid in the milk.[22]

The neonatal murine model for *G. duodenalis* infection is now well established in the medical research community and allows investigators the opportunity to examine the biology of the parasitic organism, the host-parasite relationship, and the pathogenesis of giardiasis *in vivo*. This animal model also has the potential to explore in detail the role of nutrients and dietary supplementation in mammalian host resistance (and that of their offspring) to intestinal protozoa.[23]

The dietary fatty acid compounds used as supplements in these current investigations were dodecanoic acid (DA), monolaurin (dodecanoic acid, 2, 3 dihydroxypropyl ester), and cold-pressed coconut oil. Dodecanoic acid (C:12; synonymous with lauric acid), is known to have greater antimicrobial / antiprotozoal activity than other medium-chained saturated fatty acids (MCSFA)such as caprylic acid (C:8), capric acid (C:10) or myristic acid (C:14).[24] This MCSFA is one of the most widely distributed saturated fatty acids found in nature and it was first identified in *Lauraceae* seeds (*Laurusnobilis*) by Marsson T in 1849.[24] It is predominantly found in cinnamon oil (80–90%) coconut oil (40–60% as trilaurin) and also in *Cuphea* plant species (4%) (*Umbelliferae*).[24] Dodecanoic acidis mainly obtained commercially from the nuts of distinct plant species, in particular *Cocos nucifera* (commonly) known as the coconut palm.[24]Approximately 50% of the coconut kernel oil is known to contain dodecanoic acid and coconut oil is composed of approximately 90% saturated fats. This fat content is predominantly medium-chain triglycerides, with 92% being saturated fatty acids, 6% monounsaturated fatty acids and 2% polyunsaturated fatty acids. Approximately 50% of the saturated fatty acid content is DA, 16.8% myristic acid, 8.2% palmitic acid and 8% caprylic acid.[24] The only monounsaturated fatty acid in coconut oil is oleic acid, while the only polyunsaturated fatty acid is linoleic acid[25–26]. In the mammalian[27] body, DA can be converted to its water-soluble derivative monolaurate glycerate (Monolaurin™). This derivative cannot be formed in the body *de novo* pathways.[24,28]

To date, there have been no previous investigations on the anti-parasitic effects of dodecanoic acid, coconut oil or monolaurin using the neonatal murine model for giar-

diasis. Likewise, there are no reports on the effects of dietary fatty acid supplementation on fatty acid expression in murine maternal breast milk and subsequent resistance of suckling offspring to *G. duodenalis* infection. This is a pilot study to determine the validity of possible future studies to explore the effects of dietary fatty acid supplementation of lactating maternal mammals on the intestinal parasitic burdens in their suckling offspring by using the murine model of giardiasis.

MATERIALS AND METHODS

Source and maintenance of experimental animals

Specific pathogen-free Quackenbush Swiss (outbred) timed-pregnancy female mice (*Mus musculus*) were supplied by the Animal Resources Centre, Perth, Western Australia. Mice were maintained in a pathogen-free environment at the Griffith University Animal Facility according to Australian National Health and Medical Research Council (NHMRC) guidelines for animal welfare. Sterilized tap water was offered ad libitum and lighting and heating were supplied on a regular cyclic manner to maintain animal health and well being.

Parasite culture

The *Giardia duodenalis* S-2 (sheep strain 2) trophozoite strain used in this study were previously supplied by Professor Andre Buret, University of Calgary, Canada. *G. duodenalis* tropozoites were maintained and subcultured anaerobically at 37°C in TYI-S-33 growth media supplemented with 1% bovine bile (Sigma), 10 % Serum Supreme (Cambrex Bio-products) and 200 IU/ml penicillin/200 µg/ml streptomycin (In vitrogen, USA). Confluent mid log phase cultures were pass-aged every 2 days by chilling the cultures on ice for a minimum of 10 min, followed by vortexing to dislodge the adherent trophozoites from the walls of the culture vessel. Fresh culture media (5 ml) was seeded with approximately 1×10^5 trophozoites for each passage.

Animal ethics

Animal trials were reviewed and approved by the Griffith University Animal Ethics Committee (BBS/01/03/aec). The Australian NHMRC Guidelines and Animal Care Guidelines for the Care and Use of Experimental Animals were strictly followed in all studies.

Dietary supplementation for the experimental animals

Adult, maternal mice (greater than 6 months of age) were offered supplements of dodecanoic acid, coconut oil or monolaurin in their typical daily diets, which consisted of grain pellets (wheat, lupins, barley, soya meal, fish meal, canola oil and salt) containing no dodecanoic acid or coconut oil derivatives. To 20gm of these pellets, 2gm portions of supplemental dodecanoic acid (Sigma Aldrich), monolaurin (Lauricidin®) or coconut oil (generic supermarket cold pressed organic virgin oil) were melted at 55-60°C and then evenly poured and mixed over the pellets. After cooling for one hour, the pellet meals were then offered to all mice at the same time each day (at 08.00 hours). Any leftover pellets were weighed and the average amounts of supplement consumed by the maternal mice were calculated and recorded. The negative control maternal mice were offered 20 gms of normal unaltered pellets (with no additional supplementation), at the same time of day as those receiving the supplemented diets.

Trial 1: Dodecanoic acid (DA) supplementation (0.81 g ± 0.58 g per day)

The maternal mouse was offered a diet supplemented with dodecanoic acid for 8 days [3 days ante-partum and then 5 days post-partum] and the average amount of the DA ingested was 0.81 g ± 0.58 per day. This mouse delivered a litter of 9 pups. The negative control maternal mouse was given a normal diet of 20 g grain pellets [i.e. no dodecanoic acid supplementation] per day, and housed in a separate cage at a distance from the mouse receiving the supplemented diet. The control mouse delivered a litter of 13 pups. At 3 days of age, all pups from both litters were orogastrically inoculated with 1×10^5 *G. duodenalis* trophozoites as per the standard protocol.

Trial 2: DA supplementation (0.3 ± 0.05 g per day)

This second maternal mouse was offered a diet supplemented with dodecanoic acid for 8 days (3 days ante-partum and then 5 days post-partum) and the average amount of DA ingested was 0.3 g ± 0.05 per day. This mouse delivered a relatively small litter of 4 pups. The control maternal mouse was given a normal diet (i.e. no dodecanoic acid supplementation) and delivered a litter of 14 pups. At day 3 of age, all pups from both litters were then orogastrically inoculated with 1×10^5 *G. duodenalis* trophozoites.

Trial 3: Coconut oil supplementation (1.72 g ± 0.42 per day)

The third maternal mouse was offered a diet supplemented with coconut oil for the same time as described previously. The average amount of coconut oil ingested was 1.72 g ± 0.42 per day, which gave a calculated average daily intake of 0.86 ± 0.21 g of DA. This mouse delivered a litter of 16 pups. The control maternal mouse was given a normal diet (no DA or coconut oil supplementation) for the same time period. The control mouse delivered a litter

of 14 pups. All pups were orogastrically inoculated at 3 days of age with 1×10^5 *G. duodenalis* trophozoites.

Trial 4: Monolaurin supplementation (1.18 g ± 0.32 per day)

Monolaurin™ (dodecanoic monoglycerate) was supplemented into the diet of the fourth maternal mouse for 8 days as per the other trials and the average amount of monolaurin ingested was 1.18 g ± 0.32 per day. This mouse delivered a litter of 10 pups. The control maternal mouse was offered a normal diet (i.e. no DA, monolaurin or coconut oil supplementation) for 8 days. This control maternal mouse delivered a litter of 12 pups. At day 3 of age, all pups from both litters were then orogastrically inoculated with 1×10^5 *G. duodenalis* trophozoites.

Experimental infection in neonatal murine pups

Following standard protocols, litters of mice were infected at 3 days of age by orogastric inoculation with 1×10^5 *G. duodenalis* trophozoites [S2 Strain] in 0.05 ml of cold PBS via silastic tubing (0.025 inch diameter, Dow CorningCorporation) attached to a blunted 26-gauge needle and 1 ml syringe (Terumo).

At 6 days post-inoculation, each pup was euthanized by a lethal intraperitoneal injection of 10 mg / 0.15 ml sodium pentabarbitone (Rhone Merieux Pty. Ltd). Similarly, maternal mice were also euthanized by intraperitoneal injection of 30mg of sodium pentabarbitone.

Parasite enumeration from the intestinal tract of murine pups and adults

The entire intestinal tract from the pyloric sphincter to the rectum was surgically removed from each necropsied animal. The intestinal tracts were then sliced open longitudinally, and placed in 5 ml (pups) or 20 ml (maternal adult) of cold, sterile PBS, in sterile plastic screw-top test tubes (Sigma Aldrich Nunc Australia). The tubes containing the gut sections were kept on ice for at least 30 minutes and thoroughly vortexed for 1-2 minutes three times to dislodge trophozoites from the intestinal epithelium. The mean counts of total parasite burdens [trophozoite and cyst] were calculated from at least five separate counts on the gut washes of each animal using a Neubauer haemocytometer.

Microscopy and staining techniques

In order to detect motile *Giardia* trophozoites, wet mounts of intestinal washes were prepared on clean slides (Livingstone Products) and viewed immediately under an Olympus light microscope. In some instances, smears were stained with either 20 µl of Lugol's iodine solution (ICN Biomedicals) or 0.1% toluidine blue (BDH

Chemicals Australia) in PBS to assist differentiation of trophozoites and cysts from faecal debris and commensal intestinal yeast cells. Trypan Blue dye (Sigma Aldrich) exclusive was also employed to differentiate viable from non-viable trophozoites and cysts in gut washes.

Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments. One-way ANOVA was used to calculate statistical significance between control and treated groups with a p value < 0.01 considered to be statistically significant.

RESULTS

In Trial 1, all pups of the maternal mouse that received a mean intake of 0.81 g ± 0.58 per day of dodecanoic acid were clear of any *G. duodenalis* trophozoites or cysts at necropsy at Day 6 post-inoculation. In contrast, all pups of the control maternal mouse that received a normal non-supplemented diet were heavily infected with *G. duodenalis* with a mean total parasite burden of $6.2 \pm 7.6 \times 10^5$.

In Trial 2, all pups of the maternal mouse that received a mean intake of 0.30 ± 0.05 g per day of dodecanoic acid were also clear of any *G. duodenalis* trophozoites or cysts at necropsy at Day 6 post-inoculation. All pups of the control maternal mouse that received a normal non-supplemented diet were again heavily infected with *G. duodenalis* with a mean total parasite burden of $8.6 \pm 3.5 \times 10^5$.

In Trial 3, all pups of the maternal mouse that received a mean intake of 1.72 g ± 0.42 per day of cold-pressed coconut oil [which gave a calculated average daily intake of 0.86 ± 0.21 g of dodecanoic acid] were infected with *G. duodenalis* at necropsy at Day 6 post-inoculation. The mean total parasite burden was $5.1 \pm 6.3 \times 10^5$, and both trophozoites and cysts were observed in intestinal washes. Likewise, all pups of the control maternal mouse that received a normal non-supplemented diet were very heavily infected with *G. duodenalis* with a mean total parasite burden of $18.2 \pm 11.07 \times 10^5$.

Similarly in Trial 4, all pups of the maternal mouse that received a daily mean intake of 1.18 g ± 0.32 per day of monolaurin (dodecanoic monoglycerate) were infected with *G. duodenalis* at necropsy at Day 6 post-inoculation. The mean total parasite burden was $7.0 \pm 4.03 \times 10^5$, and both trophozoites and cysts were again observed in intestinal washes. All pups of the control maternal mouse that received a normal non-supplemented diet were also

infected with *G. duodenalis* with a mean total parasite burden of $7.75 \pm 6.36 \times 10^5$.

The maternal adult mice from all trials were negative for *G. duodenalis* trophozoites or cysts, irrespective of their dietary intake or supplementation. Further studies will be performed at a later date on the collected maternal breast milk as well as the maternal blood sera to determine the fatty acid profiles of supplemented and non-supplemented maternal mice in these trials.

DISCUSSION

The aim of this research was to compare the anti-parasitic effects of dodecanoic acid to coconut oil and monolaurin (dodecanoic monoglycerate) *in vivo* using the neonatal murine model for giardiasis. It is well known that the *G. duodenalis* trophozoite employs endocytic processes to obtain nutrients and other substances such as proteins and lipids from the surrounding intestinal environment.[29,30] It
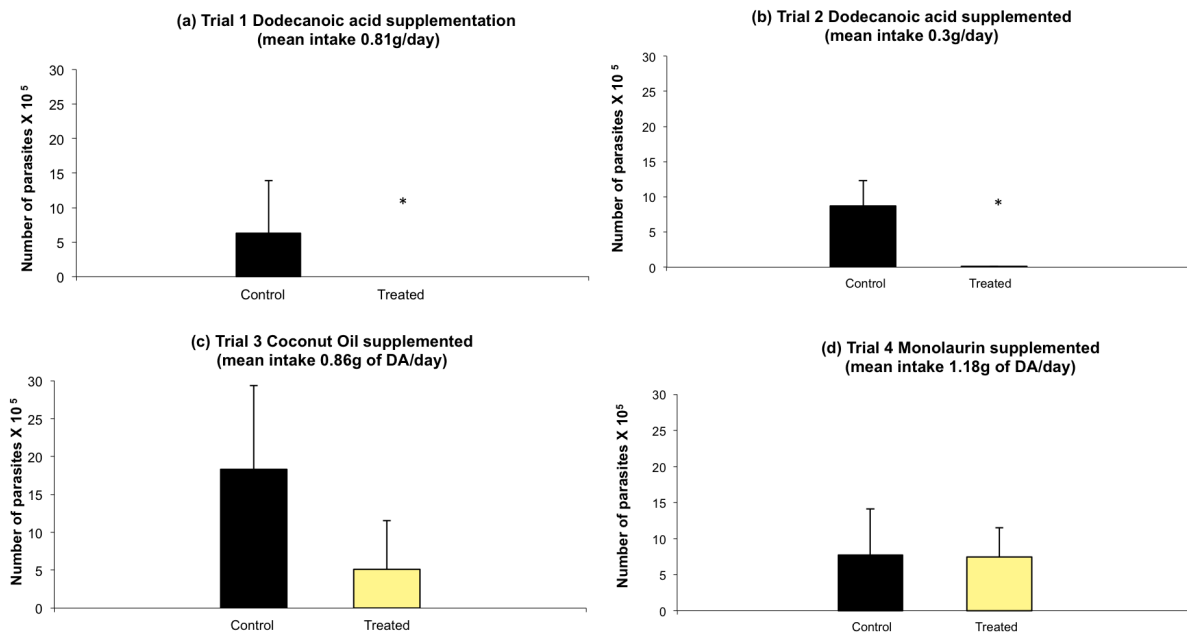

Figure 1. Mean total parasite burdens in pups at 6 days post-inoculation(a) Maternal mouse supplemented with dodecanoic acid (mean intake of0.81 gm/day) (b) Maternal mouse supplemented with dodecanoic acid (mean intake of 0.3 gm/day); (c) Maternal mouse supplemented with coconut oil (mean intake of 0.86 gm of dodecanoic acid/day); (d) Maternal mouse supplemented with monolaurin (mean intake of 1.18 gm/day); All negative control maternal mice received normal grain pellets (20 gm/day with no supplementation) (Student T- test was performed and the p value was calculated to be 0.0312).* indicates results that are significantly different to the untreated control ($p<0.01$).

is also well documented that dodecanoic acid is cytotoxic towards *G. duodenalis* trophozoites *in vitro* and has an LD_{50} that is comparable to that of metronidazole, a current drug of choice for treating giardiasis.[31] Dodecanoic acid appears to induce trophozoite death *in vitro* by accumulating in the parasite cytoplasm and forming a large lipid vacuole, which subsequently leads to the rupture of the trophozoite membrane and osmotic shock.[31]

These preliminary trials in mice using dodecanoic acid as a dietary supplement against *G. duodenalis* infection appear promising. In contrast, monolaurin and coconut oil were not effective. The ineffectiveness of monolaurin may be due to the compound's hydrophilic properties or its

glycerol moiety, which may impinge upon its ability to be absorbed through the plasma membrane of the *G. duodenalis* trophozoite.[32]

It is also well-known that the fatty acids in mammalian maternal breast milk are key components in protecting a neonate's intestinal tract from bacterial, viral, fungal and protozoal infections until the offspring's immune system has matured,[16] and one of the primary fatty acids in breast milk is dodecanoic acid.[20,21]

One possible reason for the absence of parasites in the pups of mothers offered dietary supplementations of dodecanoic acid, as well as the reduction in parasite

numbers in pups of mothers that received dietary supplementation of coconut oil, could be because a proportion of the dietary dodecanoic acid was expressed in the milk during suckling. Similar findings have been noted in other studies examining the beneficial effects of maternal breast milk on the resistance of the intestinal tracts of suckling offspring to parasitic infections.[19,33–36]

CONCLUSION

The range of drugs existing to treat *Giardia* infection is limited, the drug efficacies questionable, and the side-effects ranging from unpleasant to, at times, quite serious. Therefore, since the early 1990's there has been an on-going need for new anti-giardial treatment and prevention strategies. This preliminary study is the first to examine the effects of dodecanoic acid on *G. duodenalis in vivo* using the murine model. These trials indicate that in maternal mice receiving dietary supplementation of 0.30 - 0.81 g per day of dodecanoic acid, some beneficial response in suckling offspring against G. duodenalis infection is apparent. Coconut oil supplementation [comprising approximately 50% dodecanoic acid] also shows an effect against the infection, but does not fully eradicate the organism from the pups. monolaurin however appears to be ineffective against *G. duodenalis* despite reports that it has anti-viral, anti-bacterial and anti-protozoal properties.[37-42] The practical significance of these studies paves the way for future trials examining the effects of dodecanoic acid on giardiasis and other protozoal intestinal pathogens within a human population.

CONFLICT OF INTEREST

Nil

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