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Symbiont-derived sphingolipids regulate inflammatory responses in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Farmed fish live in association with diverse bacterial communities that produce wide arrays of metabolites. In rainbow trout, the skin and the gills are colonized by *Flectobacillus major*, a bacterium known to produce sphingolipids (SLs). The goal of this study is to evaluate the ability of *F. major* SLs to regulate rainbow trout inflammatory responses. *F. major* SLs were delivered by themselves or in combination with Freund's Complete Adjuvant (FCA), an oil-based adjuvant known to cause severe abdominal inflammation when injected to fish. Trout injected with SL + FCA showed decreased severity of FCA toxic effects including necrosis, granuloma formation and presence of oil droplets. However, inclusion of SLs in the FCA preparation did not decrease infiltration of immune cells intramuscularly at the site of injection. Intraperitoneal or intravenous delivery of *F. major* SLs resulted in increased expression of IgT, IgM and TGFβ transcripts in the gills but not the head-kidney and had no effects on IL-10 expression. These results indicate the *F. major* SLs regulate rainbow trout inflammatory responses and indicate that this compound can have important applications in farmed fish health management.

1. Introduction

Farmed fish suffer from a number of inflammatory diseases that can hamper their welfare, growth and market value. For example, soybean meal-induced enteritis (SBMIE) is a disease that causes severe inflammation in the intestine of salmonids (Booman, et al., 2018; Sahlmann, et al., 2013; Urán, et al., 2008; Urán, et al., 2009). Examples of inflammation caused by disease agents include heart and skeletal muscle inflammation (HSMI) disease in salmon (Kongtorp, et al., 2004) or *Flavobacterium psychrophylum* infections in salmonids (Nematollahi, et al., 2003). Vaccination is the most effective way to prevent disease outbreaks in finfish farming. Although some vaccines elicit strong immune responses by themselves, several studies have shown that vaccine efficacy in teleost fish is improved by using adjuvants in the vaccine preparation (Siwicki, et al., 1998; Tafalla, et al., 2013; Thim, et al., 2014). The most studied and potent adjuvants in fish are oil-based adjuvants. However, oil-based adjuvants are not approved for use in aquaculture due to the detrimental side

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effects they cause to fish, including lesions, adhesions, granulomas and inflammation (Bricknell and Dalmo, 2005; Noia, et al., 2014; Vazirzadeh, et al., 2008). Thus, finding alternative adjuvants or reducing the inflammatory side effects induced by oil-based adjuvants, such as Freund's adjuvant, would increase the sustainability of the fish farming industry.

Commensal bacteria bring many benefits to the host (Gomez, et al., 2013; Hooper, et al., 2012; Kelly and Salinas, 2017; Sepahi and Salinas, 2016) and can produce metabolites with potent biological properties (Arpaia and Rudensky, 2014; Gomez, et al., 2013; Hooper, et al., 2012; Levy, et al., 2016; Sharon, et al., 2014). Teleost fish live in symbiosis with large and diverse communities of microorganisms. In rainbow trout (*Oncorhynchus mykiss*), one of the most abundant commensal species residing in the skin and gills is *Flectobacillus major* (Lowrey, et al., 2015; Sepahi, et al., 2016), a bacterium that has the unique ability to synthesize glycosphingolipids (Batrakov, et al., 2000; Batrakov, et al., 1999). Recent studies in the mammalian gastrointestinal tract suggest that dietary sphingolipids or sphingolipids produced by bacteria can be absorbed by enterocytes in a number of ways including fusion of outer-membrane vesicles with host cell membranes (Heaver, et al., 2018). Absorbed sphingolipids are then mostly converted to free fatty acids and incorporated into triglycerides that enter the bloodstream in the form of chylomicrons (Heaver, et al., 2018) allowing for effects at distal sites. Uptake mechanisms at other tissue barriers such as the gills or skin have not been investigated, but sphingolipid-derived metabolites such as ceramide and SIP can be taken up by cells by different mechanisms including binding to G-protein coupled receptors (Blaho and Hla, 2014). Thus, although yet to be investigated, epithelial cells in the gills and skin of teleosts may uptake sphingolipids or their metabolites which could then enter the bloodstream eliciting local and distant effects.

Our laboratory has recently demonstrated that *F. major*-derived sphingolipids are key modulators of mucosal B cells in rainbow trout and that they can stimulate secretion of IgT and modulate the proportions of IgM and IgT B cells in lymphoid organs (Sepahi, et al., 2016). Yet, other biological functions of *F. major* sphingolipids remain unknown.

Previous work in mice has shown that sphingolipids produced by the intestinal bacterium *Bacteroides fragilis* have anti-inflammatory functions and regulate iNKT cells (An, et al., 2014). Thus, we hypothesized that *F. major*-derived sphingolipids may have important anti-inflammatory properties in rainbow trout and therefore can be used in aquaculture to ameliorate inflammatory processes. The aim of this study was to determine the ability of *F. major*-derived sphingolipids to induce anti-inflammatory cytokine responses and to test this compound in an adjuvant-model of inflammation in rainbow trout. Our results show that *F. major*-derived sphingolipids have both anti- and pro-inflammatory functions and that they are able to ameliorate side-effects caused by oil adjuvant injection in rainbow trout.

2. Material and Methods

2.1. Animals

Healthy adult rainbow trout (with mean body weight of 250 ± 20 g) were obtained from Lisboa Springs Fish Hatchery (Pecos, New Mexico, USA) and maintained at the University

of New Mexico Biology Animal Research Facility. Fish were acclimatized for two weeks prior to conducting any experiments. Fish were kept in a recirculation system with a 12-hour photoperiod and fed commercial pellets (Rangen, Inc) *ad libitum*. Prior to sampling, fish were anesthetized with MS-222 and bled from the caudal vein with a heparinized 3 mL syringe. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico, protocol number 16-200384-MC.

2.2. Bacterial growth and sphingolipid extractions and purification

F. major was grown in the specific culture medium ATCC® 29496™ at 25 °C for 30 h in 956 Microcycus medium. *F. major* sphingolipids were extracted using chloroform-methanol mixtures as explained in (Batrakov, et al., 2000). Non-lipid contents were then removed by chromatography on a Sephadex G-25 column (GE Healthcare) as described by (Wuthier, 1966). The cleaned fraction was resuspended in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) containing 2 nM bovine serum albumin (BSA).

2.3. In vivo administration of *F. major* sphingolipids

Rainbow trout were divided into four experimental groups, sphingolipids alone (SL), Freund's complete adjuvant (FCA, Sigma Aldrich, USA) alone, FCA + sphingolipid (FCA +SL) and DMEM-BSA alone (N=4). The SL and FCA+SL groups received a total of 1.4 µg of *F. major* sphingolipids in 50 µL. FCA was first emulsified in DMEM/BSA and then mixed 1:1 vol/vol with SL to make the FCA+SL emulsion. Trout were injected intraperitoneally (i.p) with 50 µL of each solution and were sampled 3 weeks later for gross morphology, examination of internal organs, and histology of the abdominal wall tissue. This experiment was repeated two-independent times.

For gene expression analysis, two independent experiments were conducted separately from the adjuvant experiments. The first experiment consisted of two groups (N=6) injected with either 50 µL of DMEM containing 1.4 µg of *F. major* sphingolipids combined with 2 nM BSA or DMEM containing 2 nM BSA (control) i.p. The second experiment contained the exact same experimental groups (N=6) but received an intravenous (i.v) injection instead of i.p. We had previously delivered the *F. major* sphingolipid preparation by i.v injection and observed changes in immune cell populations (Sepahi, et al., 2016). Thus, we used this route as a positive control. For applied purposes, however, or in adjuvant formulations, the i.p is more relevant and therefore we used both routes. Fish were sampled 60 h post-injection. A piece of the head kidney (HK) and gill tissue (from the center region of the second gill arch) were collected from each fish and placed in TRIzol for RNA extraction.

2.4. Gross morphology scoring

Overall gross morphology of the abdominal wall and internal visceral organs was scored using four parameters: reddening, inflammation, necrosis and adhesions. Each parameter was scored on a scale of 0–3 as explained in Table 1. Total gross morphology score was calculated by adding the individual scores from each of the four parameters for each fish.

2.5. Histology and light microscopy analyses

Eight abdominal wall samples per group were collected and fixed in 4% paraformaldehyde for 3 days at 4°C. After transferring samples to 70% ethanol, samples were processed and embedded in paraffin using routine histological procedures. Five µm-thick paraffin sections were stained with hematoxylin-eosin. Ten random fields were observed per samples and scored on a 0–3 scale for the presence of necrosis, granulomas, empty vesicles, oil masses and immune cell infiltration. A granuloma was defined as nodular structure composed of granulomatous cells surrounding an oil core. No attempt to identify the cellular composition of the granuloma was made. A score of 0 represents absence of observation whereas a score of 3 represents high abundance/presence of a particular feature. Light micrographs were observed under a Zeiss AxioSkop using the AxioVision software by two independent researchers.

2.6. RT-qPCR

Tissues were homogenized in 1ml TRIZol (Invitrogen) using tungsten carbide beads (3 mm, Qiagen) and shaking (300 times per min) as per the manufacturer's instructions for RNA extraction. The RNA pellet was washed in 80% ethanol, air-dried and resuspended in RNase-free H₂O. The RNA concentration was measured by spectrophotometry (Nanodrop ND1000, LabTech) and RNA integrity determined by electrophoresis (Agilent Bioanalyser, 2100). cDNA synthesis was performed using 500 ng of total RNA, which was denatured (65°C, 5 min) in the presence of 1 µl of oligo-dT17, 1 µl dNTP (deoxynucleoside triphosphate mix 10 mM each Promega) and RNA/DNA free water (Sigma) in a volume of 13 µl. Synthesis was carried out using 1 µl Superscript III enzyme reverse transcriptase (Invitrogen) in the presence of 5 µl of 5x first strand buffer, 1 µl 0.1 M DTT, made up to a final volume of 25 µl with water and incubated at 55°C for 1 h. The resultant cDNA was stored at –20°C. The expression of IgM, IgT, IL-10, IL-1β, TGFβ-1a, TGFβ-1b and TNF-α was measured by RT-qPCR using specific primers (Table 2). qPCR was performed using 3µl cDNA template using specific primers as explained elsewhere (Sepahi, et al., 2017). Analysis was performed using the Pfaffl method (Pfaffl, 2001).

2.7. Statistical analysis

Data are expressed as the mean ± sem. Gene expression data was analyzed by t test to identify statistically significant differences between groups. Data analysis was performed in GraphPad Prism version 5.0. One-way ANOVA and a Tukey post hoc analysis test were performed to identify statistically significant differences among groups. P values < 0.05 were considered statistically significant.

3. Results

3.1. Combination of FCA with *F. major* sphingolipids ameliorates gross morphology of adjuvant-derived tissue damage

The abdominal wall and visceral organs were inspected for inflammation, reddening, necrosis and adhesions (Fig. 1A–D). Substantially more reddening at and around the injection site was observed in FCA group, as compared to FCA+SL (Fig. 1A). Inflammation

of the abdominal wall was also observed in both FCA and FCA+SL groups, but was greater in the FCA group compared to the FCA+SL group (Fig. 1B). The muscle tissue of the abdominal wall surrounding the site of injection showed signs of necrosis in both groups but was significantly more extensive in the FCA group compared to the FCA+SL treatment (Fig. 1C). As expected, FCA i.p injection resulted in presence of adhesions throughout the entire abdominal cavity (Fig. 1D). Small adhesions of the peritoneum to the abdominal wall were observed in FCA+SL group. Injection of SL alone resulted in a mean score of 0.25 ± 0.17 (Fig. 1E and Table 3). The DMEM-BSA group (negative control) showed no signs of damage around the site of injection (0.00 ± 0.00). Reddening, inflammation, necrosis and adhesions were lower in FCA+SL group (0.97 ± 0.58) compared to FCA alone (2.5 ± 0.61) (Fig. 1E). These results indicate the *F. major* sphingolipids ameliorate gross damage caused by FCA injection.

3.2. Combination of FCA with *F. major* sphingolipids ameliorates adjuvant-derived tissue damage but causes intramuscular immune cell infiltration

Histological observation of the skeletal muscle tissue at the site of injection showed no damage or immune cell infiltration in DMEM-BSA controls (Fig. 2A). As expected, infiltrates of immune cells were observed among skeletal muscle fibers in the FCA only group (Fig. 2B). Surprisingly, the FCA+SL group showed a higher degree of immune cell infiltration compared to the FCA group (Fig. 2C). Extensive areas of necrosis were observed in the FCA only but was far less frequently observed in the SL and FCA+SL groups (Fig. 3A). Granulomas were observed most frequently and extensively in FCA group with only one granuloma observed in the FCA+SL group and no granulomas in the DMEM-BSA or SL only groups (Fig. 3B). In agreement with previous study (Noia, et al., 2014), large empty vacuoles were observed in both FCA and FCA+SL groups (Fig. 3C). The vacuoles that were observed in FCA only group were larger and surrounded by few cells. In contrast, the FCA+SL group contained smaller vacuoles that were surrounded by several cells (not shown). Within the abdominal wall section, oil masses were observed only in FCA only and FCA+SL groups, appearing to be far larger and more prevalent in FCA only group (Fig. 3D). Finally, increased deposits of melanin were only observed in the FCA group around the site of injection (not shown). Combined, these results indicate that *F. major* sphingolipids reduce the side effects caused by FCA injection, but they also stimulate infiltration of immune cells intramuscularly.

3.3. *In vivo* delivery of *F. major* sphingolipids induces changes in IgM, IgT and cytokine expression in HK and gills

I.p injection of SL resulted in a significant up-regulation of both IgM (3-fold) and IgT (5-fold) gene expression in the gills. In the HK, i.p delivery of SLs resulted in a down-regulation in IgM and IgT expression, 2-fold and 4-fold, respectively (Fig. 4A, B). Pro-inflammatory cytokine IL-1 β expression was down-regulated 2-fold in the HK but did not change significantly in gill (Fig. 4C). TNF α expression was up-regulated 1.6-fold in the gills but not in the HK of i.p SL injected group (Figure 4D). The expression of anti-inflammatory cytokine TGF β -1b was upregulated in the gills and remained unchanged in the HK (Fig. 4F). No significant changes in expression were observed for IL-10 or TGF β -1a (Fig. 4E, G) in response to i.p administration of SL.

IgM and IL10 expression remained unchanged in both HK and gills in response to i.v SL delivery (Fig. 5A, G). I.v delivery of SLs caused an up-regulation of IgT expression in the gill (Fig. 5B) but not in the HK. IL-1 β expression was significantly upregulated in the gill but not HK in response to i.v SL delivery (Fig. 5C). The expression of the pro-inflammatory cytokine TNF α was up-regulated in the HK but not the gill (Fig. 5D) Finally, anti-inflammatory cytokines TGF β -1a and TGF β -1b were both significantly up-regulated in the gill but not in the HK (Fig. 5E, F).

These results indicate that, at mucosal surfaces such as the gills, *F. major* sphingolipids stimulate IgT as well as pro-inflammatory and anti-inflammatory cytokine expression but that these effects are less pronounced systemic lymphoid tissues such as the HK compared to mucosal sites such as the gills.

4. Discussion

Host biological processes can be influenced by commensal-derived metabolites (Gomez, et al., 2013; Hooper, et al., 2012; Kelly and Salinas, 2017; Sepahi and Salinas, 2016). Host immunological responses can be directed by commensals in a variety of ways, including the induction or suppression of inflammation (An, et al., 2014; Hooper, et al., 2012). Thus far, the functions of commensal-derived metabolites such as lipids have not been exploited in the context of aquaculture. In this particular study, we evaluated the ability of *F. major*-derived sphingolipids to regulate inflammatory responses in rainbow trout.

We focused on sphingolipids derived from *F. major* since this is a prevalent commensal of the skin and gills of trout (Lowrey, et al., 2015) and we recently showed that sphingolipids isolated from this bacterium modulate rainbow trout immunoglobulin and B cell responses (Sepahi, et al., 2016). To investigate the effects of the *F. major*-derived sphingolipids in trout inflammatory responses, we paired it with the commonly used oil-based adjuvant, Freund's Adjuvant. Oil-based adjuvants paired with vaccines are widely used to help elicit effective immunological responses but are often accompanied by several adverse side effects in fish (Tafalla, et al., 2013). Specifically, Freund's Adjuvant has been shown to induce inflammation, adhesions and granulomatous lesions at the injection site and visceral organs, and has also been shown to deposit droplets of the emulsified adjuvant within the abdominal cavity (Gjessing, et al., 2012; Noia, et al., 2014; Villumsen, et al., 2015). Thus, there is a current need to develop new adjuvants or, as proposed in this study, combine FCA with other compounds that decrease its toxicity. Our findings indicate that commensal-derived sphingolipids like the one tested here are an inexpensive and promising tool to regulate inflammatory diseases in farmed fish. Injection of *F. major* sphingolipids alone did not result in leucocyte infiltration at the site of injection 3 weeks later. It is possible that leucocytes infiltrated the injection site at earlier time points and it deserves further investigation. Based on our observations, however, it is possible that the leucocyte influx elicited by FCA delivery acquires an anti-inflammatory phenotype when FCA+SL are present. A final point to consider is that vaccines formulated with sphingolipids should afford equal or better protection than the vaccine formulation without the sphingolipids. Thus, future studies will evaluate the effects of incorporating *F. major* sphingolipids with FCA in a vaccine

preparation with regards to vaccine immunogenicity, levels of protection and types of specific adaptive immune responses.

Previous studies with *F. major* sphingolipids have demonstrated the ability to stimulate secretion of IgT and modulate the proportions of IgM and IgT B cells via i.v. injection in rainbow trout (Sepahi, et al., 2016). IgT is an immunoglobulin isotype specialized in mucosal immunity in teleost fish (Salinas, et al., 2011; Tacchi, et al., 2014; Xu, et al., 2016; Xu, et al., 2013; Zhang, et al., 2010). Thus, we expected to observe greater modulation of IgT expression compared to IgM expression in our present study. In accord, we were able to demonstrate that *F. major* sphingolipids mostly stimulated IgT expression and to a lesser extent IgM expression in a mucosal tissue, the gills. Interestingly, these effects occurred in parallel to a decrease in IgT and IgM expression in systemic lymphoid tissues (HK). Given that we have previously shown that *F. major* sphingolipids orchestrate the distribution of B cells in mucosal and systemic organs, we speculate that the changes in gene expression here recorded may be due to redistribution of B cells exiting the HK and reaching the gills in response to sphingolipid treatment. Further studies will address the mechanisms by which *F. major* sphingolipids is able to regulate Ig transcript expression.

Commensals are known to influence host transcriptional programs (Brestoff and Artis, 2013; Meireles-Filho and Deplancke, 2017; Meisel, et al., 2018). Commensals, compared to pathogens, are known to induce anti-inflammatory responses in the mucosal environment (Round and Mazmanian, 2010; Sokol, et al., 2008; Tlaskalová-Hogenová, et al., 2011). Previous studies in mammals using *Bacteroides fragilis* sphingolipids identified anti-inflammatory roles for this compound. The present study tested a crude preparation of *F. major* sphingolipids that likely contains mixtures of phosphatidylethanolamine, a common molecule in teleost fish, and monoglycosyldiacylglycerol, a common molecule in most bacteria. Thus, delivery of *F. major* sphingolipids resulted in mixed pro-inflammatory and anti-inflammatory cytokine expression responses in trout. We observed different gene expression responses depending on the route of delivery (i.p or i.v). I.p injection of sphingolipids resulted in significant up-regulation of IgM, IgT, TNF α and TGF β -1b expression in the gills but down-regulation of IgM and IL-1 β in the HK. I.v delivery, in turn, stimulated a significant upregulation of TNF α in the HK and IgT, IL-1 β , TGF β -1a and TGF β -1b in the gills. We previously reported that i.v injection of *F. major* sphingolipids results in lower IgT proportions in the gills and higher IgT proportions in the HK compared to control trout (Sepahi, et al., 2016). Thus, the changes in cytokine gene expression observed in the i.v group may reflect the effects that sphingolipids have on B cells populations. Alternatively, effects on other cells types cannot be ruled out. Overall, our results highlight the potential for application of *F. major* sphingolipids in a variety of diseases and disorders in rainbow trout farming. Since we used a mixture of phosphatidylethanolamine and monoglycosyldiacylglycerol, future studies using purified fractions from this preparation in order to identify the specific compounds that trigger anti-inflammatory versus pro-inflammatory responses in fish.

Our studies have thus far only evaluated effects of *F. major* sphingolipids by injection. However, mucosal delivery of this compound either in the water or incorporated into the feed should be tested in order to facilitate larger scale applications of our results.

Additionally, we did not quantify whether sphingolipid administration exerted long-lasting effects on the local and systemic trout immune system. Future studies will address these key aspects of sphingolipid administration.

In conclusion, we have demonstrated the immunological functions of a commensal derived sphingolipid in rainbow trout. The diverse and potent effects that *F. major* sphingolipids have on rainbow trout immune and inflammatory responses suggest that this compound can have important applications in aquaculture.

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Highlights

- Intraperitoneal injection of symbiont-derived sphingolipids ameliorates FCA gross damage
- Intraperitoneal injection of symbiont-derived sphingolipids reduces necrosis, granulomas and oil masses
- Symbiont-derived sphingolipids stimulate IgT and IgM expression in gills but not head-kidney
- Symbiont-derived sphingolipids regulate TGF β expression but not IL-10 expression in gills
- I.p injection of Symbiont-derived sphingolipids decrease IL-1 β expression in the head-kidney and increase TNF α expression in the gills

Fig. 1. Gross morphology of rainbow trout abdomen 3 weeks post injection (i.p) of Freund's Complete Adjuvant combined with *F. major* sphingolipids (FCA+SL) or Freund's Complete Adjuvant alone (FCA). Pictures are representative of four different fish per group. (A) reddening of the skin at the site of injection (black arrow), (B) inflammation at the site of injection (black arrow) and (C) necrosis of abdominal tissue and (D) adhesions of the peritoneum to the abdominal wall. Total average score (arbitrary units) of all four parameters (reddening, inflammation, necrosis and adhesions) for each of the four experimental groups are plotted in (E). Results are representative of two independent experiments.

Fig. 2.

Hematoxylin and eosin staining of abdominal wall muscle tissue from rainbow trout injected i.p with DMEM (control), Freund's Complete Adjuvant (FCA), *F. major* sphingolipids alone (SL), or Freund's Complete Adjuvant containing sphingolipids (FCA+SL) and sampled 3 weeks later.

Fig. 3.

Pathology scores based on histological observation (Fig. 2) of rainbow trout (N=4) injected i.p with DMEM (control), Freund's Complete Adjuvant (FCA), *F. major* sphingolipids alone (SL), or Freund's Complete Adjuvant containing *F. major* sphingolipids (FCA+SL) and sampled 3 weeks later and sections were stained with hematoxylin-eosin. Ten random sections from each sample were scored by two independent researchers using scores of 0, 1, 2 or 3 for (A) cellular necrosis; (B) granulomas; (C) vacuoles; (D) oil masses; and (E) immune cell infiltration. Results are representative of two independent experiments.

Fig. 4.

Rainbow trout (N = 6) were injected i.p with *F. major* SLs combined with BSA in DMEM or BSA in DMEM alone. Fold-change expression of (A) IgM; (B) IgT; (C) IL-1 β ; (D) TNF α ; (E) TGF β -1a; and (F) TGF β -1b; (G) IL-10 were measured in the Head Kidney (HK) and gills 60 h post-treatment by RT-qPCR. Bars represent mean fold change $\pm$ standard error. Asterisks denote statistically significant differences (*p < 0.05, **p < 0.01).

Fig. 5.

Rainbow trout (N = 6) were injected i.v with *F. major* SLs combined with BSA in DMEM or BSA in DMEM alone. Fold-change expression of (A) IgM; (B) IgT; (C) IL-1 β ; (D) TNF α ; (E) TGF β -1a; and (F) TGF β -1b; (G) IL-10 were measured in the Head Kidney (HK) and gills 60 h post-treatment by RT-qPCR. Bars represent mean fold change $\pm$ standard error. Asterisks denote statistically significant differences (*p < 0.05, **p < 0.01).

Table 1

Gross morphology scoring system (arbitrary units)

Damage	Score
Reddening: no superficial reddening Inflammation: no inflammation Necrosis: no visible necrosis Adhesions: no adhesions of peritoneum to abdominal wall or visceral organs	0
Reddening: minor superficial reddening Inflammation: minor inflammation (only visible superficially) Necrosis: minor reddening and inflammation within affected tissue Adhesions: minor adhesions of peritoneum to abdominal wall (not affecting visceral organs)	1
Reddening: moderate superficial reddening Inflammation: moderate deep and superficial inflammation Necrosis: moderate reddening and inflammation within affected tissue Adhesions: moderate adhesions of peritoneum to abdominal wall/visceral organs	2
Reddening: severe superficial reddening Inflammation: severe deep and superficial inflammation Necrosis: severe reddening and discoloration around affected tissue Adhesions: severe adhesions of peritoneum spanning entire abdominal wall or adhering to visceral organs	3

Table 2

Primers used for RT-qPCR study

Gene Symbol	Accession Number	Sequence (5' → 3')
Ef1a	AF498320	F: CAACGATATCCGTCGTGGCA R: ACAGCGAAACGACCAAGAGG
IgM	OMU04616	F: AAGAAAGCCTACAAGAGGGAGA R: CGTCAACAAGCCAAGCCACTA
IgT	AY870264	F: CAGACAACAGCACCTCACCTA R: GAGTCAATAAGAAGACACAACGA
IL-10	NM_001245099.1	F: CTGCTGGACGAAGGGATTCTAC R: GGCCTTTATCCTGCATCTTCTC
IL-1 β	AJ223954	F: ACATTGCCAACCTCATCATCG R: TTGAGCAGGTCCTTGTCCTTG
TGF1 β – 1a	AJ007836	F: CTCACATTTTACTGATGTCACCTCCTGT R: GGACAACCTGCTCCACCTTGTG
TGF1 β – 1b	FN822750	F: CATGTCCATCCCCAGAACT R: GGACAACCTGTTCCACCTTGTGTT
TNF- α	AJ277604	F: GGGGACAACTGTGGACTGA R: GAAGTTCTTGCCCTGCTCTG

Table 3

Tabulated mean and standard deviation for each parameter.

Groups	Damage	Mean	Standard Deviation
DMEM/BSA	Reddening	0.00	0.00
	Inflammation	0.00	0.00
	Necrosis	0.00	0.00
	Adhesions	0.00	0.00
SL only	Reddening	0.00	0.00
	Inflammation	0.25	0.43
	Necrosis	0.75	0.43
	Adhesions	0.00	0.00
FCA + SL	Reddening	0.50	0.50
	Inflammation	1.25	0.33
	Necrosis	1.25	0.43
	Adhesions	1.00	0.71
FCA only	Reddening	2.37	2.37
	Inflammation	2.50	0.50
	Necrosis	3.50	0.50
	Adhesions	3.62	0.62