Chapter 6

Impact of Low Glucose Degradation Products Bicarbonate/Lactate-Buffered Dialysis Solution in an uremic mouse Peritoneal Dialysis exposure model

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ABSTRACT

Background
High Glucose Degradation Products (GDPs) content, acidic pH and presence of lactate characterize conventional peritoneal dialysis fluids and are known to mediate peritoneal membrane deterioration. Bicarbonate/lactate solutions with neutral pH and glucose degradation product content have been reported to offer advantages in terms of inflammation and incidence of peritonitis. Nevertheless, not all peritoneal changes related to bicarbonate/lactate solutions exposure are considered advantageous so some controversial issues remain.

Methods
We used our recently developed uremic Peritoneal Dialysis (PD) mouse exposure model to perform a screening of peritoneal markers for fibrosis, vascularisation as well as inflammation. Mice underwent 5/6 nephrectomy and were daily instilled with standard lactate, bicarbonate/lactate-buffered solutions or saline during a period of 8 weeks. Histological analysis for membrane thickening, myofibroblasts and peritoneal macrophages influx was carried out on the parietal peritoneum. Vasculature was quantified in omentum. Peritoneal cell populations and cytokines expression were determined in peritoneal effluents by flow cytometry analysis and ELISA respectively.

Results
We showed that low GDPs bicarbonate/lactate-buffered solution prevented peritoneal increase of α-SMA and CD31 expression. It induced pro-inflammatory macrophage recruitment and an enhanced peritoneal expression of pro-inflammatory cytokines such as TGFβ-1, TNF-α and IL-1β. In addition, in our study inflammatory mechanisms resulted to be mediated by Th17 and Th1 rather than by Th2 cell populations.

Conclusions
Compared to standard lactate PD fluid, a more biocompatible buffered PD solution preserved peritoneal membrane from PD-related angiogenesis and fibrosis. However, this treatment resulted in inflammatory responses, which might be mainly related to the recruitment of pro-inflammatory macrophages in the peritoneum.
CONTINUOUS AND LONG-TERM EXPOSURE OF THE PERITONEUM TO PERITONEAL DIALYSIS FLUIDS (PDFs) IS ASSOCIATED WITH INFLAMMATORY MECHANISMS AS EPITHELIAL TO MESENCHYMAL TRANSITION (EMT), PERITONEAL ANGIGENESIS, THICKENING OF THE PERITONEAL MEMBRANE AND OTHER CHANGES OF THE PERITONEAL MEMBRANE RESULTING IN TECHNIQUE FAILURE AND CRITICAL CLINICAL OUTCOMES. ALL THESE EVENTS SEEM TO BE DRIVEN MAINLY BY THE HIGH GLUCOSE CONCENTRATION, THE HIGH GLUCOSE DEGRADATION PRODUCTS (GDPs) CONTENT, THE LOW pH AND THE PRESENCE OF LACTATE THAT CHARACTERIZE CONVENTIONAL PDFs 1. IN THE LAST DECADeS MORE BIOCOMPATIBLE PDFs HAVE BEEN DEVELOPED AND BROUGHT INTO THE CLINICAL PRACTICE. LACTATE-BICARBONATE BUFFERED SOLUTIONS WITH MORE PHYSIOLOGICAL pH IN WHICH THE AMOUNT OF GDPs HAS BEEN LOWERED HAVE BEEN INTRODUCED AND ALTERNATIVE SOLUTIONS IN WHICH GLUCOSE HAS BEEN REPLACED BY ALTERNATIVE OSMOTIC AGENTS SUCH AS ICODEXTRIN AND AMINO-ACIDS HAVE BEEN DEVELOPED IN ORDER TO PRESERVE THE DIALYTIC FUNCTION OF THE PERITONEAL MEMBRANE 2. PDFs HAVE BEEN ALSO SUPPLEMENTED WITH NEW GENERATION COMPOUNDS AS REVIEWED BY FARHAT ET AL 3. IN THIS RESPECT WE RECENTLY SHOWED THAT ALANYL-GLUTAMINE DIPEPTIDE PROTECTS AGAINST PD INDUCED PERITONEAL FIBROSIS AND ANGIoGENESIS 4.

THE INTRODUCTION OF NEUTRAL pH AND PARTICULARLY BICARBONATE/LACTATE-BUFFERED SOLUTIONS SEEMED TO OFFER ADVANTAGES IN TERMS OF PERITONEAL MEMBRANE PRESERVATION AND PERITONEAL HOMEOSTASIS CONTROL 5. IN THE EURO BALANCE TRIAL IT DEMONSTRATED A SIGNIFICANT IMPROVEMENT IN EFFLUENT MARKERS OF PERITONEAL MEMBRANE INTEGRITY, A DECREASE IN SYSTEMIC ADVANCED GLYCATION END PRODUCTS (AGEs) LEVELS, LESS DECLINE IN RESIDENT RENAL FUNCTION AND A DECREASE IN PERITONEAL ULTRAFILTRATION 6. MOREOVER, SUCH BIOCOMPATIBLE FLUIDS SHOWED TO DELAY THE ONSET OF ANURIA INCREASE MESOTHELIAL CELL MARKERS, CAUSE LESS SYSTEMIC INFLAMMATION AND REDUCE THE INCIDENCE OF PERITONITIS 7-9. EX VIVO STUDIES ALSO SHOWED THAT THE pH AND LACTATE HAVE THE MOST PROFOUND EFFECT ON CELL FUNCTION WITH A BETTER PRESERVATION OF BACTERIAL CLEARING CAPACITY 10.

GDPs ARE THOUGHT TO BE THE MAIN FACTORS IN PDFs RESPONSIBLE FOR PERITONEAL MEMBRANE DETERIORATION. THESE HIGHLY REACTIVE SUBSTANCES HAVE SHOWN TO INDUCE EPITHELIAL TO MESENCHYMAL TRANSITION (EMT) IN VITRO 11 AND EX VIVO 12, CAUSE MESOTHELIAL INJURY AND REDUCE MESOTHELIAL REGENERATION THUS INCREASING THE RISK FOR PERITONEAL FIBROSIS 13. THE USE OF LOW GDPs FLUIDS CAN PREVENT PERITONEAL FIBROSIS AND VASCULAR SCLEROSIS BY SUPPRESSING AGE ACCUMULATION 14. MOREOVER, IT RESULTED IN PRESERVATION OF MESOTHELIAL CELL MASS AND PERITONEAL TRANSPORT 15, 16.

WITHIN OUR GROUP WE HAVE ANALYZED THE EFFECT OF A PD REGIMEN LOW IN GLUCOSE AND GLUCOSE DEGRADATION PRODUCTS IN BOTH ANIMAL AND CLINICAL STUDIES. THE DATA INDICATED BETTER PRESERVATION OF PERITONEAL MORPHOLOGIC FEATURES UPON BICARBONATE/LACtATE SOLUTION (BLS) EXPOSURE IN THE RAT MODEL BUT INFLAMMATORY CHANGES WERE UNSTUDIED DUE TO UNAVAILABILITY OF ADEQUATE MARKERS 10. MOREOVER, IN HUMAN STUDIES SOME CONTROVERSIAL FINDINGS REGARDING PERITONEAL ACTIVATION AND INFLAMMATORY CYTOKINES WERE REPORTED WHICH REMAIN UNEXPLAINED 16. TO FURTHER INVESTIGATE ON THIS REGARD,
the recently developed uremic mouse PD exposure model \cite{17} was used in the present study to compare a neutral low-GDP bicarbonate/lactate-buff ered solution with a standard high GDPs lactate PDF in respect of inflammation, fibrosis and vascularisation. The uremic model more closely mimics the clinical situation of a patient undergoing PD and gives the chance to study the effect of PDFs exposure in concomitance with peritoneal and systemic changes caused by uremia. Moreover, since more reagents and antibodies are available in mouse, the mouse model offers advantages over the previous rat models, to study PD-related fibrosis and inflammation pathways. In addition, novel peritoneal markers identified in the current study and the mouse model itself, might be used in the future to knock out genes playing a crucial role in the study of PDF-induced maladaptive peritoneal changes.

**RESULTS**

In the present report we focused on important indicators for peritoneal inflammation and tissue remodelling during peritoneal repair such as fibrosis and angiogenesis. The uremic status of the mice was confirmed by a 2 fold increase in both urea and creatinine levels (data not shown), which closely mimics the conditions of a kidney patient undergoing PD. Mice were randomized into three different treatment groups and daily exposed to saline (S) or one of the two glucose-based PDFs among Dianeal (lactate-buff ered solution indicated as LS) and Physioneal (Bicarbonate/Lactate-buff ered solution indicated as BLS). A healthy control not treated group was also included (C).

During the treatment no animal dropped out due to one of the treatment regimens and no differences in body weight were observed indicating lack of toxicity for both of the two PDFs.

**Peritoneal cell output and ultrafiltration**

Analysis of the peritoneal effl uents collected after 8 weeks of daily exposure to the different treatments performed by standard peritoneal equilibrium test (PET) showed non-significant differences between the groups regarding ultrafiltration (UF) (data not shown).

Moreover, total number of peritoneal cells measured in those effl uents increased in both the PDF groups compared to control group. The percentage of total T-cells decreased after PD but no significant changes in both CD8 and CD4 positive populations were found while the significant increase in CD4 / IL-17 double positive lymphocytes (CD4+ IL-17+) registered in the LS group was restored in the BLS group (** P=.007). No significant differences were seen between the groups neither in the percentage of B cells nor monocytes (Ly6G+ CD11b+), although the number of the latest increased especially after BLS. Similarly to previous studies in mice \cite{4} a slight
increase in macrophages (F4/80+ CD11b+) was already shown after exposure to conventional LS and this rise became clearly significant in the BLS group (* P=.03) (table 1). So exposure to BLS clearly gives profound changes over LS in terms of peritoneal inflammatory cells recruitment.

**Exposure to low GDPs bicarbonate/lactate-buffered solutions can induce pro-inflammatory macrophages recruitment.**
Further analysis by immunohistochemistry suggested that the increase in macrophages noticed in the effluents was related to an increased recruitment of F4/80+ subset in the parietal peritoneum of the mice exposed to BLS. Indeed both the CD11b / F4/80 double positive and the F4/80 single positive populations were significantly increased in the BLS group compared to both the control and the LS groups reflecting a pro-inflammatory M1 phenotype \(^{18}\). On the other hand, staining for Dectin-1 as marker for anti-inflammatory M2 macrophages did not reveal any significant differences among groups. (figure 1).

**Exposure to low GDPs bicarbonate/lactate-buffered solutions does not prevent peritoneal thickening but decrease angiogenesis and fibrosis in the uremic mouse PD model**
Our previous studies showed that fibrosis and thickening of the peritoneal membranes were taking place in the uremic mouse model after exposure to conventional PD fluid \(^{17}\).
After 8 weeks of daily exposure to the PDFs a significant increase in peritoneal thickness compared to the non-PDF-exposed control and saline exposed groups was shown (C: 30.63±1.83, S: 41.84±4.91, LS: 78.78±16.17, BLS: 78.11±12.42; P=0.02). However, thickening of peritoneum was not differently influenced by different PDF compositions (LS or BLS) (figure 2c).

![Image](image1.png)

**Table 1.** Composition of cells (in percentage) in mouse peritoneal effluents after 8 weeks of exposure to saline (S), conventional (LS) or low GDPs bicarbonate/lactate buffer (BLS). Control (C) indicates the not-PD-exposed group.
Figure 1. Effect of low GDPs bicarbonate/lactate-buffered solutions on pro-inflammatory macrophages recruitment. Panel (a) shows representative peritoneal membrane macrophages recruitment for each group in the uremic PD mouse model (C: control, S: saline, LS: lactate PDF, LBS: lactate/bicarbonate PDF). Peritoneal staining for CD11b (violet), F4/80 (red), CD11 / F4/80 double positive and Dectin-1 (green) are represented in the rows from the top to the bottom panel respectively. Nucleus are stained with DAPI (blue). Column bars represent number of macrophages measured per field in three different pictures taken per mouse per each group (n=10). Significant increase of CD11b / F4/80 double positive and F4/80 positive cells in the BLS group are represented respectively in graphs (b) and (c). Graph (d) shows Dectin-1 positive cells in each group (C: white bar, S: black bar, LS: square bar, LBS: striped bar). (* P<.05). Magnification 20x.
Conversely, immunohistological analysis of peritoneal biopsies revealed that exposure to BLS resulted in a significant reduction of α Smooth Muscle Actin (αSMA) positive cells accumulating in the parietal membrane upon daily instillation with conventional LS PDF (C: 0.02±0.00, S: 0.02±0.01, LS: 0.06±0.02, BLS: 0.01±0.00; P=.03) (figures 2a and 2b). Furthermore, the significant increase of new vessel formation in the omentum, represented by the up-regulation of Cluster of Differentiation 31 (CD31) protein in the LS group when compared with the control group, was reduced after BLS exposure (figure 3a and 3b). So with respect to fibrosis and angiogenesis, the harmful effect caused by LS treatment is prevented by BLS exposure.

Analysis of effluent cytokines and biomarkers
In order to further investigate peritoneal inflammation, we performed a screening of the main inflammatory cytokines expressed in the effluents of our mice after the treatment. Our measurements at the protein level showed an increase of Transforming Growth
Factor β 1 (TGFβ1) upon both PD treatments (C: 27.33±16.06, LS: 204.1±94.70, BLS: 536.9±81.54; P=0.009) (figure 4a). A similar result was obtained regarding Interleukin-1β (IL-1β) that was already significantly up-regulated in the LS group and further increased upon BLS exposure (C: 12.88±0.80, LS: 17.64±3.23, BLS: 40.94±10.15; P=0.01) (figure 4b).

It is known that TGFβ1 and IL-1β act in synergy to enhance Interleukin-6 (IL-6) production 19. In our study IL-6 release increased as a consequence of PDF exposure but it seemed not to differ between the PD regimens (C: 68.06±22.06, LS: 2070±855.1, BLS: 1729±783.7) (figure 4c). A remarkable increase of Tumor Necrosis Factor α (TNF-α) was also shown after PD exposure and it was most prominent after BLS exposure (C: 10.38±0.62, LS: 33.57±9.3, BLS: 210.3±98.93; P=0.02) (figure 4d). The differences between the two alternative PD treatments encountered in CD4+ IL-17+ cell population were not reflected by a significant increase in Interleukin-17 (IL-17) production, although lower protein levels of this cytokine were found in the BLS group (figure 4e).

Figure 3. Effect of bicarbonate/ lactate buffered PDF on peritoneal angiogenesis. (a-b) Immunofluorescence microscopy analysis of omentum sections stained with CD31 marker shows accumulation of vessels in the group exposed to LB (bicarbonate fluid: square bar) and protection from angiogenesis in the BLS group (striped bar). Each value corresponds to an average (% surface staining CD31) of 10 independent values of each rat omentum taken each time. Cholm bars represent data as means ± SE of at least 6 animals per group. (*) *P<.05. Magnification 20x.
We found increased levels of Interleukin-5 (IL-5) in the LS group which did not occur upon treatment with BLS (C: 18.44±1.2, LS: 53.40±14.80, BLS: 23.44±4.56) (figure 4f). The same was found for Interleukin-4 (IL-4) (C: 13±0.35, LS: 73.36±30.46, BLS: 18±2.7) (figure 4g), indicating involvement of T helper 2 (Th2) cells only upon LS treatment and not BLS exposure.

On the other hand, significant high levels of Interferon γ (INFγ) (figure 4h) detected only after BLS exposure suggested that in BLS regimens an important role may be played by T helper 1 (Th1) cells (C: 23.69±2.82, LS: 42.18±11.42, BLS: 184.8±57.88; P=0.005).

Furthermore, a significant rise of Macrophages Inflammatory Proteins 1α and 1β (MIP1α and MIP1β also known as CCL3 and CCL4) appeared as a consequence of BLS treatment (respectively for MIP1α C: 46.19±1.7, LS: 84.82±14.23, BLS: 420±223.8 and MIP1β C: 12.81±0.57, LS: 53.43±18.80, BLS: 278.8±110.4; P=0.02) (figures 4j and 4k), which coincides very well with the observed macrophage (and especially pro-inflammatory M1)-influx after BLS (table 1, figure 1).

**DISCUSSION**

The current study is the first one using a mouse PD exposure model comparing lactate and bicarbonate/lactate-buffered solutions in a uremic setting, which more closely mimics the clinic status of a PD patient. Moreover, due to the availability of more reagents in the mouse, our study provides a more extensive analysis of the peritoneal and omental morphological alterations and a more accurate screening of inflammatory and tissue remodelling parameters when compared to previous animal studies.

Our results showed that the use of low-GDP bicarbonate/lactate PDF did not result in a less impaired ultrafiltration as was also shown in our recent study in both rats and mice on PD-exposure 4. This fact, together with the less peritoneal thickening obtained in animal models after exposure to new generation PD treatments, indicates a reduction in their harmful effect.

Although in our uremic mouse PD model the exposure to BLS did not prevent thickening of the peritoneal membrane, we showed that a low GDPs bicarbonate/lactate regimen can be more effective than a conventional PD treatment in preserving from αSMA positive cells accumulation in the parietal peritoneum, indicating a protective role of BLS against fibrosis. Moreover, daily exposure to BLS rather than LS prevented new vessel formation which may indicate a beneficial effect of the BLS regimen in terms of peritoneal angiogenesis. In this regard, previous independent studies suggested that the use of low GDP solutions was associated with less αSMA expression in a rat model 11 and bicarbonate/lactate buffered regimen correlated with reduced angiogenesis and fibrosis 20.

The introduction of neutral pH and particularly bicarbonate/lactate solutions might
Figure 4. Exposure to a bicarbonate/lactate buffered solution is associated with increase of pro-inflammatory cytokines. Protein levels of the main pro-inflammatory cytokines detected in effluents collected from mice after 8 weeks of PDF exposure. Cytokines levels (pg/ml) are represented as means ± SE as follow: (a) TGFβ1, (b) IL-1β, (c) IL-6, (d) TNFα, (e) IL-17, (f) IL-5, (g) IL-4, (h) INFγ, (j) MIP1α, (k) MIP1β. Column bars represent data as means ± SE of 5 animals per group. (* P<.05, ** P<.01).
better preserve the function of peritoneal membrane cell populations. In our study exposure to both PDF did not affect the total cell number measured in the peritoneal effluents collected after the 8 weeks of treatment, neither their cell composition except for a significant decrease in the number of CD4+ IL17+ cells and increase in (pro-inflammatory M1) macrophages expression only in the BLS group. Although the increase of CD4+ IL17+ upon LS treatment is in line with previous studies in mouse, the significant reduction of this population in the BLS group was not reflected by a significant decline of the IL-17 protein levels. On the other hand, the increase in the percentage of macrophages cell population in the peritoneal effluents was associated to an enhanced recruitment of pro-inflammatory M1 macrophages in the peritoneal membrane. Our results showed indeed accumulation of macrophages in the parietal peritoneum and prevalence of the pro-inflammatory (F480+) over the anti-inflammatory subset (Dectin-1+) in the BLS group. Moreover in the same group, protein levels of both MIP1α and MIP1β, important chemotactic factors for macrophages, were increased. Similarly, also IL-1β and TNFα, both pro-inflammatory cytokines produced by macrophages were higher in the BLS group validating the hypothesis of an increased pro-inflammatory macrophages influx upon BLS treatment.

We furthermore revealed a significant rise of TGFβ1 levels together with IL-1β in the BLS group when compared to the group undergoing standard PD treatment. TGFβ1, as TNFα, is known to be pro-inflammatory cytokines playing an important role during tissue fibrosis, infection and in the onset of chronic disease state. Nevertheless, the role of TGFβ1 is still controversial and its pleiotropic effects are context dependent.

The comparison between the conventional and biocompatible PD treatment also showed significantly higher expression of INFγ in the effluents in the BLS group and higher levels of IL-5 and IL-4 in the LS group. This findings suggested that inflammatory mechanisms occurring during exposure to bicarbonate/lactate buffer involve Th1 rather than Th2 cell subset while the opposite happened upon exposure to high GDPs lactate solutions.

Taken together these results might contradict the hypothesis of lower inflammatory effect upon biocompatible solutions. Nevertheless, in some PD studies increased levels of TNF-α, TGFβ1 and INFγ have been interpreted as a consequence of improved mesothelial and macrophages cell function instead to be seen as part of a pro-inflammatory process. In our opinion, this effect might rather indicate an inflammatory process led by an influx of M1-macrophages.

It has been shown that in the inflammatory phase newly attracted macrophages present a more pro-inflammatory (M1) phenotype but only after the switch to M2 they become pro-fibrotic. Moreover, we previously showed in vitro that only M2 macrophages, and not M1, secrete factors inducing α-SMA expression and EMT. Recently it was also established in a clinical study that PD-related fibrosis correlate with an increase in M2 macrophages. So, based on these findings, our present
results showing prevalence of peritoneal M1 over M2 macrophages should not be interpreted as an harmful effect of BLS exposure. To conclude, the results in this uremic mouse PD model suggested that the use of bicarbonate/lactate low GDPs buffered solutions leads to better preservation of the peritoneal membrane in terms of fibrosis and angiogenesis. Moreover, our study showed that upon exposure to BLS decreased fibrosis and vascularization are mainly associated with peritoneal recruitment of M1 macrophages, higher levels of macrophages-related pro-inflammatory cytokines and lower number of CD4+/IL-17+ cells. So we interpret the inflammatory changes in cell population after BLS exposure as positive, although further analysis need to be performed in order to investigate the expression of those markers at the systemic level and to establish the exact mechanism involved.

**METHODS**

**Mouse PD model**

*C57BL/6J female mice* (Charles River, Maastricht, The Netherlands) aged 12-14 weeks and weighing approximately 20 g at the start of the study were used. Animals were organized as follow: 1 healthy control group (n=10), 3 PD groups (n=10 per group) daily exposed to 2ml saline or standard lactate-buffered solution (Dianeal®, Baxter) or bicarbonate/lactate-buffered solution (Physioneal®, Baxter) during a period of 8 weeks. Mice in all the PD groups underwent 5/6 nephrectomy and catheter implantation (Customized mouse catheter MMP-4S-061108A, Access Technologies, Ridgeway, USA). 5/6 nephrectomy consisted in the complete removal of the right kidney and the removal of the anterior and posterior 1/3 part of the left kidney by using a monopolar electric blade as described previously described 17. All the animals were housed under standard conditions and were given food and water *ad libitum*. Health conditions were checked daily. The experimental protocols were approved by the Animal Welfare Committee at the VU University Medical Center, Amsterdam.

**Cell counting**

At the end point, following the injection of 2ml standard PDF via catheter, peritoneal effluents were collected after 30 min, cells were isolated by centrifugation and counted and stained with fluorochrome-conjugated mouse-specific antibodies against CD3, CD4, CD8α, B220, CD11b, Ly6C, F4/80 and IL-17 purchased from eBiosciences. Before intracellular staining, cells were re-stimulated for 4 h with 50ng/ml Phorbol 12-Myristate 13-Acetate (PMA) and 500ng/ml ionomycin in the presence of 1μg/ml BD Golgi Plug (eBiosciences). Samples were analysed in a BD FACS Fortessa (BD Biosciences) flow cytometer and further analyses were performed with FloJo software.
Histology and Immunohistochemistry

Parietal peritoneal biopsies were collected from the opposite side from the catheter installation. The biopsies were fixed in Bouin’s solution, embedded in paraffin, cut into 5 μm sections and stained with Masson’s Trichrome. Peritoneal membrane thickness was determined using a Carl Zeiss Microscope (GmbH, 37081, Göttingen, Germany). Microscope photographs were obtained by using an AxioCam ICc5. Peritoneal thickness of each animal was calculated by the median of measurement taken every 50 μm from one side to the other of the tissue sample.

Biopsies were frozen in Tissue-Teck® (O.C.T.® Sakura) and cut into 5 μm sections. To identify myofibroblasts and vessels samples were stained for anti-rat Alpha Smooth Muscle Actin (αSMA 1A4, DAKO, 1:500) combined with anti-mouse-IgG (H+L) (Invitrogen) and Cluster of Differentiation 31 (αCD31, PECAM, Serotec, Oxford, UK, 1:1000) coupled to anti-mouse-IgG-555 according to the manufacturer’s instructions. Nuclei were stained with DAPI. Fluorescence microscopy was performed with a Carl Zeiss Microscope and photographs were taken with an AxioCam HR R3. The areas positive for CD31 were calculated by CellProfiler software (2.1.1, Broad Institute, UK).

Quantification of cytokines

Peritoneal effluents collected after 8 weeks of treatment were made cell free by centrifugation (300G, 5mins, RT) and stored at -20°C. Protein levels of mouse TGFβ1, IL-1β, TNFα, IL-17A, IL-6, INFγ, IL-5, IL-4, MIP1α and MIP1β were quantified by ProcartaPlex™ Multiplex Immunoassays (affymetrix eBioscence).

Statistical analysis

Data were analysed using GraphPad Prism software (La Jolla, CA). Statistical analysis was performed using One-way ANOVA test to compare the groups. Bonferroni’s test was used for multiple comparisons. A P-value <0.05 was considered statistically significant (’*’ = P<.05, ’**’ = P<0.01, ’***’ = P<.001). Data were shown as means ± SEM.

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