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RESEARCH

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# Effect of lactate as a peritoneal dialysis fluid buffer on rat peritoneal mesothelial cells



Chieko Higuchi<sup>\*</sup> , Junko Kuriyama and Hiroshi Sakura

## Abstract

**Background:** Neutral, low-glucose degradation product (GDP) peritoneal dialysis fluid (PDF) is less damaging to the peritoneum than conventional PDF but is still insufficient for biocompatibility. One remaining issue is the problem of buffering.

**Methods:** Using cultured rat peritoneal mesothelial cells (PMCs), the present study examined the difference between the effects of neutral low-GDP lactate PDF and neutral low-GDP bicarbonate/lactate PDF on cells. The effects of lactate stimulation on these cells were also examined.

**Results:** Lactate PDF enhanced mRNA expressions of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) and type 1 and type 3 collagens and lowered expression of e-cadherin mRNA in PMCs compared to bicarbonate/lactate PDF. Lactate stimulation increased mRNA expressions of  $\alpha$ SMA, matrix metalloproteinase 2 (MMP2), and basic fibroblast growth factor (bFGF) and suppressed e-cadherin mRNA expression. Transforming growth factor (TGF)- $\beta$ 1 and TGF- $\beta$ 2 and collagen type 1 and 3 mRNA expressions were also enhanced by lactate stimulation.

**Conclusions:** These results suggest that lactate as a PDF buffer may act on PMCs to promote epithelial-mesenchymal transition (EMT) and production of TGF- $\beta$ , bFGF, and collagen.

**Keywords:** Peritoneal dialysis fluid, Buffer, Lactate, Peritoneal mesothelial cell, Epithelial-to-mesenchymal transition, Fibrosis, Transforming growth factor- $\beta$ , Basic fibroblast growth factor

## Background

Peritoneal dialysis (PD) is an established treatment in renal replacement therapy, can be performed at home without bulky equipment, and can maintain a high quality of life. However, long-term PD treatment causes pathological changes to the peritoneum, such as detachment of mesothelial cells, fibrosis of the interstitium, increases in the new blood vessels, and narrowing of the vascular cavities [1]. These changes result in peritoneal dysfunction, making PD difficult to maintain. The risk of complications such as encapsulating peritoneal sclerosis due to peritoneal disorders is also increased [2]. To address this problem, a change has been made from acidic high-GDP PDF to

neutral low-GDP PDF, achieving a reduction in rates of peritoneal disorders [3]. However, some problems remain with the biocompatibility of these new PDFs, with the issue of buffering representing one such problem. In Japan, all buffering agents for neutral low-GDP PDF to date have been lactate, but a bicarbonate/lactate buffer PDF has recently been released. However, few studies have examined the effects of buffering agents on the peritoneum. This study investigated the effects of neutral low-GDP PDF containing different buffers on the peritoneum and further clarified the effects of lactate on the peritoneum.

## Methods

### PDF

Dianeal N<sup>®</sup> (Baxter, Tokyo, Japan) was used as the lactate low-GDP PDF, and Reguneal<sup>®</sup> (Baxter) was used as the

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bicarbonate/lactate low-GDP PDF. Table 1 shows the constituents of these two PDFs.

### Culture of PMCs

The isolation and culture of PMCs were performed according to our previously reported method [4]. Briefly, omentum was obtained from the abdominal cavity of Sprague-Dawley rats and dissolved in 0.05% trypsin and 0.02% ethylenediaminetetraacetate solution at 37 °C for 20 min. Cellular components were then isolated by centrifugation (150×g, 3 min) and washed with phosphate-buffered saline (PBS). Cells were suspended in Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F12 (DMEM/F12) medium (Thermo Fisher Scientific, Yokohama, Japan) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Cromwell, CT), penicillin at 50 U/mL, and streptomycin at 50 µg/mL. Cells were seeded into 10-cm<sup>2</sup> collagen type I-coated Petri dishes at 37 °C under a 5% CO<sub>2</sub>/95% air atmosphere. PMCs were identified by the uniform cobblestone appearance at confluence, by negative staining for von Willebrand factor, and by positive staining for both cytokeratin and vimentin. Confluent cultures at the third passage were used in experiments.

### Examination of two different PDFs and two different buffers

Confluent cultured PMCs were divided into 5 groups: control group, L-PDF group, B/L-PDF group, L group, and B/L group. The control group used cultured mesothelial cells in DMEM/F12 + 10% FBS. The L-PDF and B/L-PDF groups used cultured cells in Dianeal N<sup>o</sup> or Reguneal<sup>o</sup> + 200 mM glutamate (Wako Pure Chemical Industries, Osaka, Japan) (both with the same content of DMEM/F12) + 10% FBS. The L and B/L groups used cultured cells in PBS + 40 mM lactate (Wako Pure Chemical Industries) or 30 mM NaHCO<sub>3</sub> (Wako Pure Chemical Industries) with 10 mM lactate + 200 mM glutamate + 10% FBS. All culture media were adjusted to pH 7.0 using HCl. Cells were stimulated in these media for 72 h.

**Table 1** Constituents of lactate PDF and bicarbonate/lactate PDF

|                          | Lactate PDF | Bicarbonate/lactate PDF |
|--------------------------|-------------|-------------------------|
| pH                       | 6.5–7.5     | 6.8–7.8                 |
| Na (mEq/L)               | 132         | 132                     |
| Ca (mEq/L)               | 3.5         | 3.5                     |
| Mg (mEq/L)               | 0.5         | 0.5                     |
| Cl (mEq/L)               | 96          | 100                     |
| Lactate (mEq/L)          | 40          | 10                      |
| HCO <sub>3</sub> (mEq/L) | –           | 25                      |
| Glucose (w/v%)           | 1.36        | 1.36                    |
| Plasticizer              | –           | –                       |

### Examination of effects of lactate on PMCs

Confluent cultured PMCs were divided into 6 groups: control, L5, L10, L20, L40, and L80 groups. The control group used cultured mesothelial cells in PBS + 200 mM glutamate with 10% FBS. The L5, L10, L20, L40, and L80 groups used cultured cells in PBS + 200 mM glutamate with 10% FBS, with the addition of 5 mEq/L, 10 mEq/L, 20 mEq/L, 40 mEq/L, or 80 mEq/L of lactate, respectively. All culture media were adjusted to pH 7.0 using HCl. Cells were stimulated in these media for 6, 24, 48, and 72 h.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using NucleoSpin RNA (Macherey-Nagel, Düren, Germany) according to the instructions from the manufacturer. The cDNA was constructed with a SuperScript III for RT-PCR kit (Thermo Fisher Scientific, Yokohama, Japan). For amplification, qRT-PCR reaction solutions used 50 ng of cDNA, 10 µM of each primer, and 10 µL of FastStart Universal SYBR Green Master (Roche Molecular Diagnostics, Rotkreuz, Switzerland) in a volume of 38 µL with 30–35 cycles of 15 s at 95 °C and 15 s at 60 °C. Primer sequences are indicated in Table 2. The mRNA expression of each gene was represented by the ratio to GAPDH mRNA expression.

### Statistical analysis

Data are presented as mean ± standard deviation. All statistical analyses were performed using the Kruskal-

**Table 2** Primer sequences used in this study

| Gene              | 5'-3'                     |
|-------------------|---------------------------|
| GAPDH-F           | CTAGAGACAGCCGCATCTTCTT    |
| GAPDH-R           | CAATGTCCACTTTGTCCACAAGAGA |
| αSMA-F            | GATCACCATCGGGAATGAACGC    |
| αSMA-R            | TGTTATAGGTGGTTTCGTGGATGC  |
| e-cadherin-F      | GAGAGTCGAGGTGCCTGAGG      |
| e-cadherin-R      | TCCAAATCCGATACGTGATCTTCTG |
| MMP2-F            | GGGACAAGTTCTGGAGATACAATGA |
| MMP2-R            | ACTTCACACTCTCAGACTTTGGT   |
| TGFβ-1-F          | CCC CACTGATAC GCCTGA GT   |
| TGFβ-1-R          | CGA AAGCCCTGTATTCCGTCTC   |
| TGFβ-2-F          | TCAGACACACAACACCAAAGT     |
| TGFβ-2-R          | AATGTAGTAGAGGATGGTCAGTGG  |
| bFGF-F            | GAGAAGAGCGACCCACACG       |
| bFGF-R            | GTAACACACTTAGAAGCCAGCAG   |
| Collagen type 1-F | AGCCTACATGGACCAACAGACT    |
| Collagen type 1-R | CAAGTCCGGTGTGACTCGTG      |
| Collagen type 3-F | CTAGAGGATGGTCTCACTAAACAC  |
| Collagen type 3-R | TCTATGATGGGGAGTCTCATGGC   |

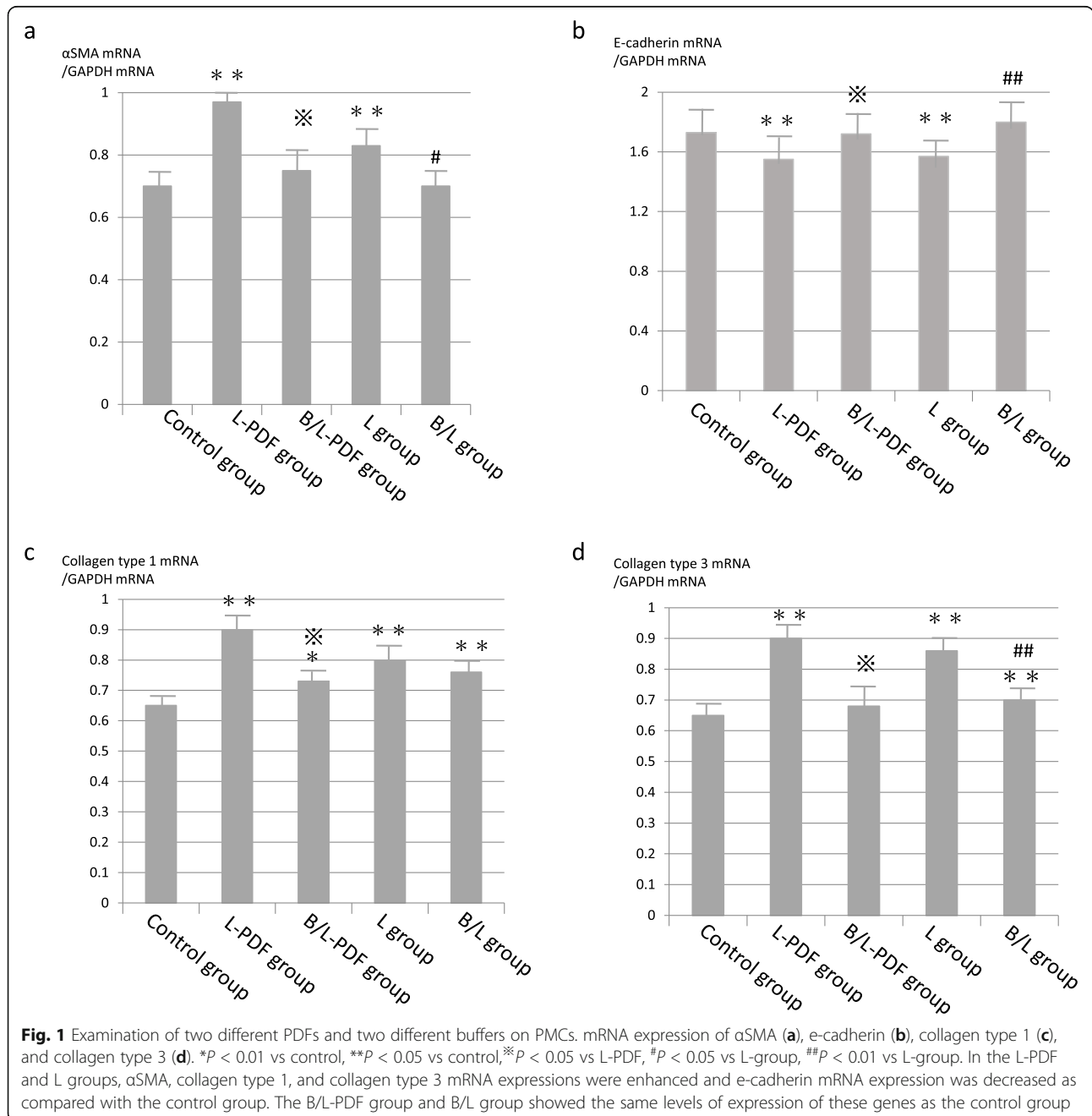
Wallis test, two-way analysis of variance, and Tukey's honestly significant difference test on JMP Pro version 14.0 software (SAS Institute Japan, Tokyo, Japan). Values of  $P < 0.05$  were considered significant.

**Results**

**Effects of two different PDFs and buffers on PMCs**

Expressions of mRNAs for  $\alpha$ SMA and collagen types 1 and 3 were higher in the L-PDF group than in the control group. Expression of e-cadherin mRNA was lower

in the L-PDF group than in the control group. However, in the B/L-PDF group, expressions of  $\alpha$ SMA, e-cadherin, and collagen type 3 mRNAs did not differ significantly from those of controls. All gene expressions in the B/L PDF group were significantly different from those in the L-PDF group. Similarly, mRNA expressions of these genes in the L group were significantly different from those in the control group, but changes in mRNA expressions for the B/L group were small and differed from those in the L group (Fig. 1).



**Effects of lactate on PMCs**

Changes in each gene expression for the L5–L80 groups according to the stimulation time of 6–72 h are represented as the ratio to gene expression in the control group. Expressions of all genes showed significant interactions between the dose factor of lactate and the factor of stimulation time. Multiple differences were thus used to examine differences in stimulus time for each stimulus dose of 5–80 mEq/L and the difference in dose for each stimulus time (6–72 h). Gene expressions for each group are shown in Figs. 2, 3, and 4 for each stimulation time and each stimulation dose.

Expression of e-cadherin mRNA decreased in a dose-dependent manner after 24, 48, and 72 h of stimulation with lactate (Fig. 2a). In the L20, L40, and L80 groups, mRNA expression decreased more with increasing stimulation time (Fig. 2d).

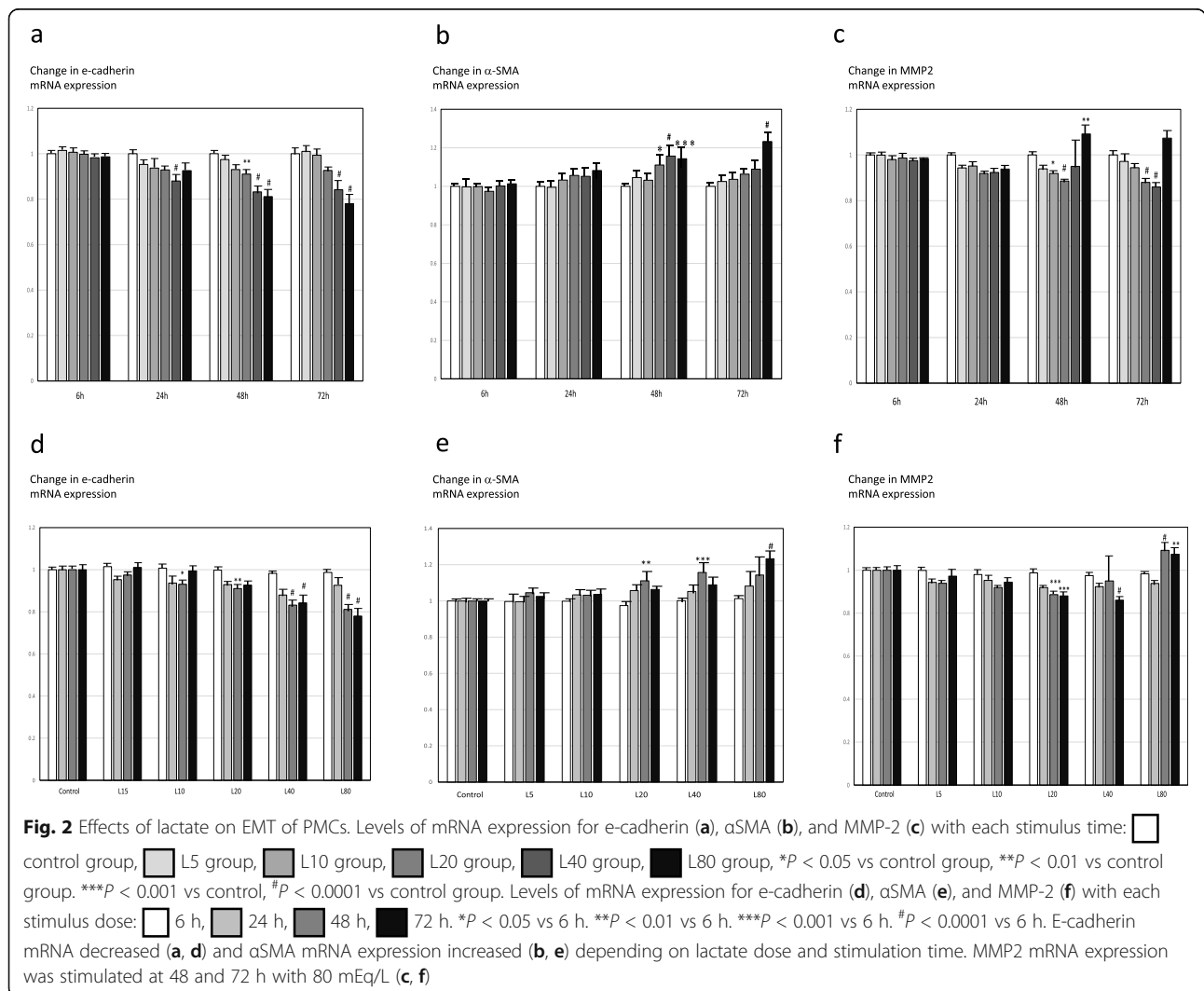
Dose- and time-dependent upregulations of  $\alpha$ SMA mRNA expression were recognized after 48 and 72 h of

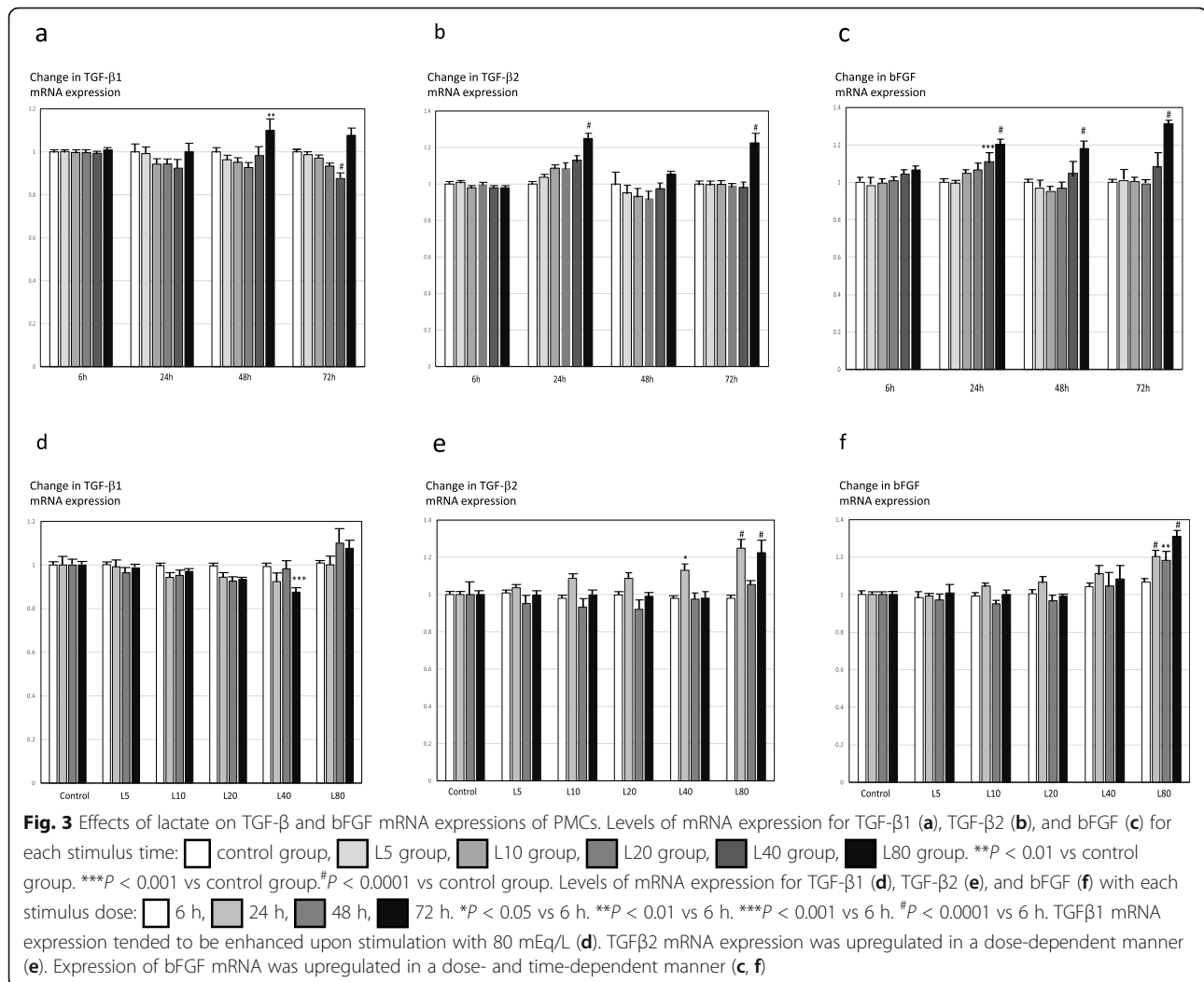
stimulation (Fig. 2b, e). MMP2 mRNA expression tended to decrease with dose at 24, 48, and 72 h of stimulation, but increased with 80 mEq/L and 48 and 72 h of stimulation (Fig. 2c).

Expression of TGF- $\beta$ 1 mRNA was increased in the L80 group at 48 and 72 h after stimulation, but no increases were seen in the other groups (Fig. 3a). TGF- $\beta$ 2 mRNA expression tended to increase with increasing dose at 24 h stimulation, but no changes were seen for other stimulation times except with 80 mEq/L stimulation (Fig. 3b). In the L80 group, TGF- $\beta$ 2 mRNA expression was increased at 24 and 72 h after stimulation (Fig. 3e).

Expression of bFGF mRNA was accelerated in a dose-dependent manner (Fig. 3c) and increased in a time-dependent manner in the L80 group (Fig. 3f).

Expression of collagen type 1 and 3 mRNA also tended to increase dose- and time-dependently with lactate stimulation (Fig. 4).



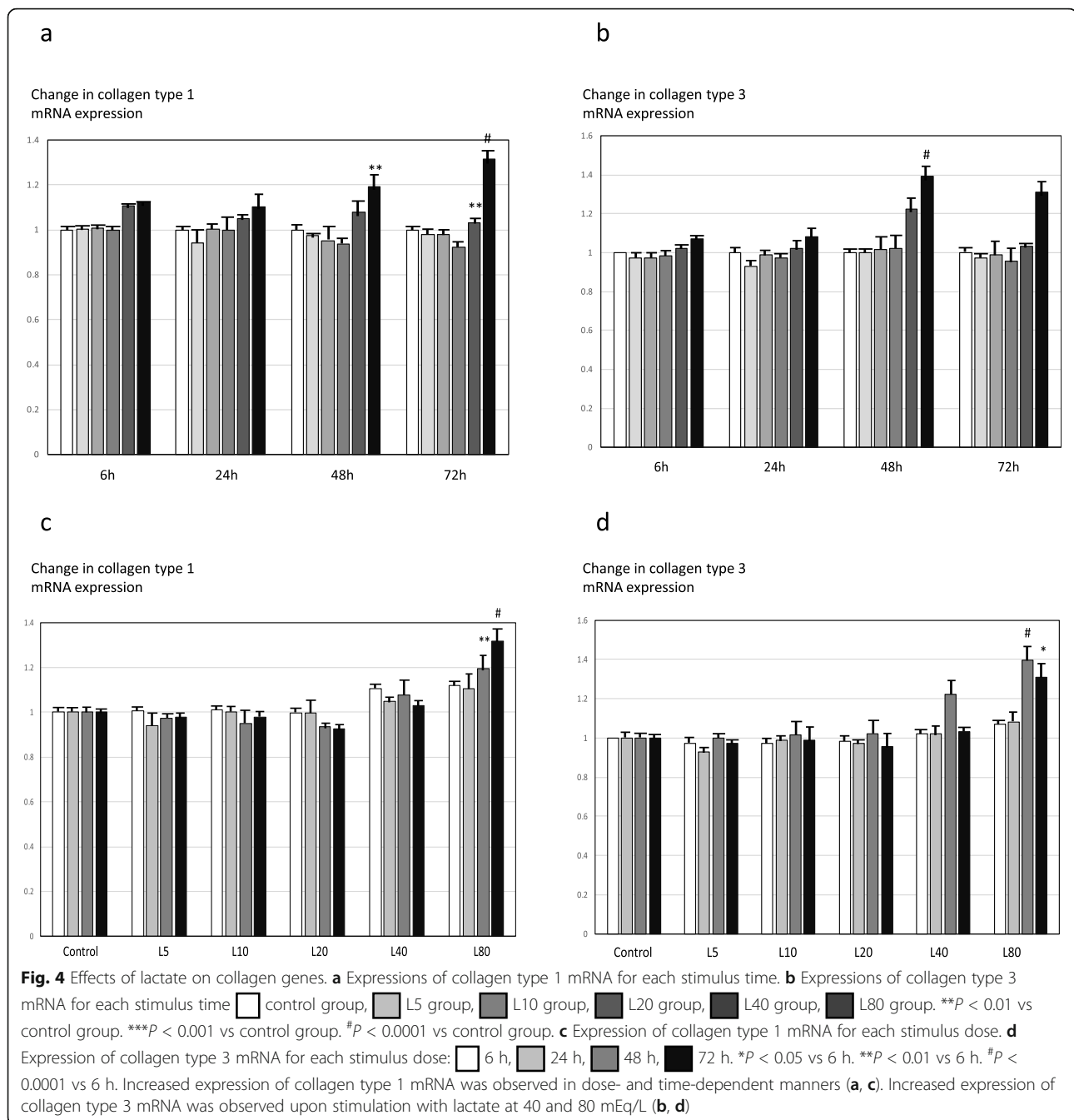


## Discussion

In the peritoneum of long-term PD patients, mesothelial cells degenerate and disappear, and interstitial fibrotic thickness and neovascularization have been reported [5]. These changes were affected by the PDF, and recognition of this fact led to the development of a biocompatible, neutral, low-GDP PDF. Many reports have shown that this new PDF results in less damage to the peritoneum than conventional acidic high-GDP PDF [3, 6, 7]. However, even this neutral, low-GDP PDF does not prevent all pathological changes to the peritoneum [8]. One of the causes is considered to be the effect of the buffer. Various reports have examined the effect of the buffer in PDF on the peritoneum, but most such studies have involved comparisons between conventional lactate PDF as a high-GDP PDF and neutral bicarbonate/lactate PDF as a low-GDP PDF [9–11]. Differences in the effects of these dialysates on the peritoneum, thus cannot be attributed to the buffer alone.

In Japan, neutral, low-GDP lactate PDF has been on the market for about 15 years, and all glucose dialysates have now been replaced with this solution. In addition to neutral, low-GDP lactate PDF, neutral low-GDP bicarbonate/lactate PDF was recently launched in Japan. The effect of the buffer in the PDF on the peritoneum was considered able to be examined by comparing these two PDFs. In the present study, the L-PDF group showed higher expression of  $\alpha$ SMA and type 1 and 3 collagen mRNA and lower expression of e-cadherin mRNA in PMCs compared to the B/L-PDF PDF group. These phenomena were also observed in comparisons between the L-group and B/L group. Based on these results, lactate PDF may enhance EMT formation and collagen production by PMCs.

One report used the same two PDFs we used and found that bicarbonate/lactate PDF was less toxic to cell viability and apoptosis of PMCs than lactate PDF [12]. In addition, a clinical study [13] reported that Fibrin



degradation products and vascular endothelial growth factor concentrations in effluent were lower when using bicarbonate/lactate PDF than when using lactate PDF. From our results and those reports, we consider that bicarbonate/lactate PDF may result in less peritoneal damage than lactate PDF.

We next examined the effects of lactate on EMT and collagen production in PMCs. Lactate decreased e-cadherin mRNA expression and increased  $\alpha$ SMA mRNA expression in a dose- and time-dependent manner. MMP2 mRNA expression was increased with 80 mEq/L

lactate at 48 and 72 h of stimulation. Increases in expressions of  $\alpha$ SMA and MMP2 mRNAs and decreases in e-cadherin mRNA expression are phenomena occurring in EMT [14], suggesting that lactate may promote EMT in PMCs.

In this study, lactate at high doses promoted TGF- $\beta$ 1 and TGF- $\beta$ 2 mRNA expressions. In addition, bFGF mRNA expression was accelerated in dose- and time-dependent manners by lactate stimulation. Few studies have examined the effects of buffer alone on peritoneal cells [15, 16]. Ogata et al. [15] reported the effects of

lactate, bicarbonate/lactate, and bicarbonate on human mesothelial cells, fibroblasts, and vascular endothelial cells. They showed that the highest amount of bFGF was produced by lactate, followed in order by bicarbonate/lactate and bicarbonate. They concluded that lactate acts on PMCs to increase bFGF production. Our results also showed that lactate increased expression of bFGF mRNA, similar to their results. TGF- $\beta$  and bFGF are known facilitators of EMT [17]. Our results suggest that lactate may activate TGF- $\beta$  and bFGF production and cause EMT.

Expressions of collagen type 1 and type 3 mRNAs were also stimulated in dose- and time-dependent manners by lactate. EMT is greatly involved in tissue fibrosis, and lactate may cause EMT in PMCs to increase collagen production and promote peritoneal fibrosis.

Many papers have described lactate produced by lactate dehydrogenase as triggering EMT in several cancer cells [18–20]. Several studies have also examined the relationships between lactate, EMT, and fibrosis [21–23]. In those reports, lactate was considered to enhance the production of TGF- $\beta$ , leading to enhanced EMT and collagen production. Among these studies, Kottmann et al. [21, 22] attributed the lactate-enhancing effects of EMT to the low pH, whereas Zareie et al. [24] showed that lactate PDF was associated with stronger angiogenesis and enhanced fibrosis regardless of pH. They concluded that the action of lactate was unrelated to pH. Our experiments standardized the pH at 7.0 to remove the need to account for the effects of pH. As a result, lactate was seen to increase EMT, TGF- $\beta$ , bFGF, and collagen production. We therefore believe that these effects are unrelated to pH, with lactate itself leading to the promotion of EMT, promotion of collagen production, and peritoneal fibrosis. In this study, only mRNA expressions were measured as markers for the effects of lactate on EMT and collagen expressions, but measurement at the protein level is also necessary, and such studies are planned for the future. Signal pathways that cause EMT of TGF $\beta$  and bFGF have been known to involve smad2/3 and snail. We believe that further research about signal pathways including smad2/3 and snail is needed on this point.

In the experiment that changed the dose and timing of lactate and examined mRNA expressions of MMP2, TGF $\beta$ 1, and collagen type 3, no significant increases were observed with 40 mEq/L lactate alone, but only at 80 mEq/L. However, changes in EMT and fibrotic mRNA were observed by stimulation with PDF containing 40 mEq/L lactate. We considered that these differences may be influenced by the GDP contained in PDF, as even neutral PDF contains GDP, albeit at low concentrations. We hope to investigate in closer detail the effects of 40 mEq/L lactate + GDP on mesothelial cells in the future.

In the mechanism of peritoneal fibrosis, mesothelial cells have been considered an important source of myofibroblasts through the EMT. However, Chen et al. [25] reported that submesothelial fibroblasts are major precursors of peritoneal myofibroblasts using an in vivo model. Lua et al. [26] have been pointed out that only 17% of myofibroblasts were derived from mesothelial cells in peritoneal fibrosis. The fibrogenic cells have not been identified with certainty. We think further consideration is needed in this regard.

## Conclusions

Lactate PDF enhanced expressions of  $\alpha$ SMA and collagen mRNA and lowered expression of e-cadherin mRNA in peritoneal mesothelial cells more strongly than lactate/bicarbonate PDF. Lactate showed an EMT-promoting effect and TGF $\beta$ , bFGF, and collagen production-enhancing effects on mesothelial cells. These results suggest that lactate in PDF may have harmful effects on PMCs.

## Abbreviations

GDP: Glucose degradation product; PDF: Peritoneal dialysis fluid; PMCs: Peritoneal mesothelial cells;  $\alpha$ SMA:  $\alpha$ -Smooth muscle actin; MMP2: Matrix metalloproteinase 2; bFGF: Basic fibroblast growth factor; TGF- $\beta$ : Transforming growth factor- $\beta$ ; EMT: Epithelial mesenchymal transition; PD: Peritoneal dialysis; PBS: Phosphate-buffered saline; DMEM/F120: Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F12; FBS: Fetal bovine serum; qRT-PCR: Quantitative real-time polymerase chain reaction

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Not applicable in this section.

## Authors' contributions

CH made substantial contributions to the study conception and design and performed the experiment, acquisition of the data, analysis, and interpretation of the data. JK performed the entire experiment. HS helped to draft the manuscript. The authors have read and approved the final manuscript submitted for publication.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Ethics approval and consent to participate

All animal experiments were conducted in compliance with the protocol, which was reviewed by the Institutional Animal Care and Use Committee and approved by the Ethics Committee of Tokyo Women's Medical University (Permit Numbers: AE-18-039, AE-19-030).

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.



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