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Original Research Article

Spermine protects intestinal barrier integrity through ras-related C3 botulinum toxin substrate 1/phospholipase C-γ1 signaling pathway in piglets

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ABSTRACT

Weaning stress can cause tight junctions damage and intestinal permeability enhancement, which leads to intestinal imbalance and growth retardation, thereby causing damage to piglet growth and development. Spermine can reduce stress. However, the mechanism of spermine modulating the intestinal integrity in pigs remains largely unknown. This study aims to examine whether spermine protects the intestinal barrier integrity of piglets through ras-related C3 botulinum toxin substrate 1 (Rac1)/phospholipase C-γ1 (PLC-γ1) signaling pathway. In vivo, 80 piglets were categorised into 4 control groups and 4 spermine groups (10 piglets per group). The piglets were fed with normal saline or spermine at 0.4 mmol/kg BW for 7 h and 3, 6 and 9 d. In vitro, we investigated whether spermine protects the intestinal barrier after a tumor necrosis factor α (TNF-α) challenge through Rac1/PLC-γ1 signaling pathway. The in vivo study found that spermine supplementation increased tight junction protein mRNA levels and Rac1/PLC-γ1 signaling pathway gene expression in the jejunum of piglets. The serum D-lactate content was significantly decreased after spermine supplementation ($P < 0.05$). The in vitro study found that 0.1 μmol/L spermine increased the levels of tight junction protein expression, Rac1/PLC-γ1 signaling pathway and transepithelial electrical resistance, and decreased paracellular permeability ($P < 0.05$). Further experiments demonstrated that spermine supplementation enhanced the levels of tight junction protein expression, Rac1/PLC-γ1 signaling pathway and transepithelial electrical resistance, and decreased paracellular permeability compared with the NSC-23766 and U73122 treatment with spermine after TNF-α challenge ($P < 0.05$). Collectively, spermine protects intestinal barrier integrity through Rac1/PLC-γ1 signaling pathway in piglets.

1. Introduction

Early life stress can lead to intestinal imbalance and growth retardation, thereby damaging piglet growth and development (Wijtten et al., 2011). Intestines are important organs for immunity, digestion and absorption, and their growth and development are closely related to the modulation of barrier function (Wen et al., 2014). The damage on intestinal tight junctions can cause intestinal integrity injury (Rao, 2008) and lead to increased intestinal mucosal permeability, cell apoptosis and necrosis (Farhadi et al., 2003). Nutritional modulation can decrease intestinal damage and maintain intestinal integrity (Ziegler et al., 2003).

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Spermine is widely distributed in animals and plants. Thus, it is found in all human foods and animal feeds. Feeds such as cereals, fish meal, and dairy products contain spermine. Spermine is one of the essential molecules in biological processes and plays an important role in maintaining normal gastrointestinal function in animals. It can increase cell proliferation and differentiation (Pegg, 2014). The intestinal barrier maintains intestinal integrity and health by preventing the infiltration of toxic substances into surrounding tissues containing occludin, claudin, zonula occludens (ZO) and myosin light-chain kinase (MLCK) (Van and Anderson, 2004). In Cdx2-transfected IEC-6 cells, the synthesis rates of tight junction decrease with polyamine content (Guo et al., 2005). The digestion, transport of nutrients, physical barrier and maintenance of large intestinal microbial homeostasis in piglets can be enhanced by spermine (Liu et al., 2020a, 2020b). However, the roles of spermine administration on transepithelial electrical resistance (TER) and paracellular permeability of the intestinal epithelial cells barrier of piglets have not been examined. The tumor necrosis factor α (TNF-α) injury model is a typically used cytokine injury model. However, the protection roles of spermine on the intestinal epithelial cells barrier of piglets induced by TNF-α have not been reported and thus need further study.

Intestinal mucosa restoration in weaned piglets includes the proliferation of crypt cells, partial recovery of villus height, regulation of brush margin enzyme activity, migration of cells and reconstruction of tight junctions (Lizuka and Konno, 2011). Cell migration plays a vital role in the restoration of epithelial cells. Ras-related C3 botulinum toxin substrate 1 (Rac1) and phospholipase C-γ1 (PLC-γ1) are the key proteins that modulate cell migration (Rao et al., 2008). Putrescine maintains rat intestinal epithelial migration by modulating the Rac1/PLC-γ1 signaling pathway (Rao et al., 2008; Ray et al., 2003). Whether spermine protects the integrity of the intestine through the Rac1/PLC-γ1 signaling pathway in pigs remains unclear and thereby requires further study.

In this study, proinflammatory cytokine TNF-α was used to construct an intestinal epithelial barrier injury model. The objective of this study was to examine the regulation of spermine on intestinal epithelial integrity and its involvement in the maintenance of epithelial integrity through the Rac1/PLC-γ1 signaling pathway.

2. Materials and methods

The animal procedures were authorised by the Care and Use of Laboratory Animals of Sichuan Agricultural University.

2.1. Animal experiments

The experimental design and diet preparation procedures were similar to those in our previous study (Fang et al., 2016). Eighty healthy suckling piglets (Duroc × Landrace × Yorkshire) were weaned at 9 d old and fed with a milk-based diet for 2 d before starting the experiment. The piglets (12 d old, 3.27 to 3.33 kg) were randomly assigned to 8 groups (10 piglets per group). Four groups of spermine-supplemented piglets were provided with adequate nutrients and supplemented with spermine (Sigma, USA; 0.4 mmol/kg BW; spermine was dissolved in water and placed into formula milk when used) in formula milk (Table S1), which was available ad libitum. The piglets were fed once a day, and they were fed for 7 h and 3, 6 and 9 d (groups SP-7h, SP-3d, SP-6d, and SP-9d, respectively). The control groups were provided with the same formula milk but with normal saline once a day for 7 h and 3, 6 and 9 d (groups Con-7h, Con-3d, Con-6d and Con-9d, respectively).

All piglets appeared well during the study. The piglets were sacrificed at the end of the feeding experiments. At the time points 7 h, 3, 6 and 9 d, the piglets were aged 12, 15, 18 and 21 d, respectively. The jejunum was immediately removed. Dose and test days of spermine supplementation were selected in accordance with previous experiments (Kaouass et al., 1996; Deloyer et al., 2005; Cheng et al., 2006; Cao et al., 2015; Liu et al., 2015).

2.2. Determination of serum D-lactate

The specific determination steps were conducted in accordance with the kit (purchased from Shanghai Enzyme Biotech Co., Ltd.) specification.

2.3. Cell culture

IPEC-J2 cell experimental protocol was performed as per our previously depicted study (Mo et al., 2021). Briefly, cells were grown in DMEM-F12 and 10% fetal bovine serum (Gibco, USA). They were pre-incubated with NSC-23766 (160 μmol/L) or U73122 (3 μmol/L) in the culture media for 1 h before spermine (0.1 μmol/L) was added to the culture medium for 50 h. The cells were pre-incubated with spermine (0.1 μmol/L) in the culture media for 2 h before TNF-α (40 ng/mL) was added to the culture medium for 48 h. Cell samples were collected after receiving 40 ng/mL TNF-α challenge for 48 h. Inhibitor of Rac1 and PLC-γ1 (NSC-23766 and U73122) were purchased from Selleck Chemicals. TNF-α (*Sus scrofa*) were purchased from Raybiotech (America).

2.4. Measurement of epithelial barrier

In this study, TER and paracellular permeability detection procedures were carried out as depicted by Watari et al. (2015). Each TER measurement was carried out by subtracting the resistance value of filters and fluids. In order to make a comparison between conditions, TER was normalized to the initial value. Flux of fluorescein-5-isothiocyanate (FITC)-conjugated dextran (FITC-dextran, 4 kDa; sigma) across monolayers was employed to represent the paracellular permeability of intestinal epithelial barrier to uncharged macromolecules. Briefly, monolayers were washed with PBS, and 200 μg/mL FD4 (FITC-dextran, 4 kDa, Sigma) was added to the epithelial chamber. Then, monolayers were incubated at 37 °C for 2 h. A 100-μL sample was taken from the basal chamber and measured by a fluorescence microplate reader. FITC-dextran flux was normalized to the control.

2.5. Real-time polymerase chain reaction (PCR) analysis

PCR experimental procedure was performed as per our previously depicted study (Mo et al., 2021). The primers of some genes in this study are listed (Table S2).

2.6. ELISA assay

The Rac1 and PLC-γ1 protein concentrations were measured using ELISA kit as per our previously depicted study (Mo et al., 2021). The specific determination steps were performed based on the kit instructions (Shanghai Enzyme Biotech Co., Ltd.).

2.7. Automated capillary Western blot

A capillary-based Western blotting system (Protein Simple Wes, San Jose, CA) was used to assess the tight junction protein expression. All procedures were completed based on the manufacturer's instructions and default settings. Rabbit-anti-actin 1:50 (Cell Signaling Technology; cat#4970s), rabbit-anti-occludin 1:50 (Abcam; cat#ab31721), Rabbit-anti-ZO-1 1:100 (Thermo Fisher Scientific; cat#61-7300), and Rabbit-anti-claudin1 1:100 (Thermo Fisher Scientific; cat#51-9000), primary antibody, the anti-rabbit

secondary antibodies included in the Wes Detection Module kit (ProteinSimple; DM-001) and 12 to 230 kDa Wes Separation Module, 8 × 25 capillary cartridges (ProteinSimple; SM-W004) were employed. All data were analysed with the Compass Software associated with the Wes instrument (ProteinSimple Wes).

2.8. Statistical analysis

Statistical analysis for all data was carried out using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). The homogeneity of variances for data was investigated by Levene's tests. For the in vivo experiment, 2 × 4 factorial data were tested as a two-way ANOVA using the general linear model procedure. The factors were spermine (0 or 0.4 mmol/kg BW) and treatment time (7 h or 3, 6, 7 d). The main effects in this study contained spermine level, treatment time and their interaction. If at least one main effect or interaction was statistically significant, the differences were determined by Duncan's multiple range tests among the treatments at $P < 0.05$. For the in vitro experiment, the data were subjected to ANOVA.

3. Results

3.1. Effect of spermine on the content of serum D-lactate in piglets

Table 1 shows that spermine supplementation significantly decreased the content of serum D-lactate by 26.02% ($P < 0.05$).

3.2. Gene expression of barrier function in the jejunum of piglets

Table 2 shows that spermine supplementation significantly enhanced tight junction mRNA levels ($P < 0.05$), significantly reduced the mRNA levels of *MLCK* ($P < 0.05$). The 3- and 6-d supplementation of spermine increased *ZO*-1 mRNA level (SP-3d vs. Con-3d; SP-6d vs. Con-6d). Spermine supplementation for 6 d significantly increased *occludin* and *claudin*-3 mRNA levels (SP-3d vs. Con-3d). The 3- and 6-d supplementation of spermine significantly enhanced *ZO-1*, *occludin* and *claudin*-2 mRNA levels, i.e., SP-3d vs. SP-7h, and SP-6d vs. SP-7h ($P < 0.05$). The 6-d supplementation of spermine significantly enhanced *claudin-1* and *claudin*-3 mRNA levels, i.e., SP-6d vs. SP-7h ($P < 0.05$). The 3-d supplementation of spermine increased *claudin*-14 mRNA level, i.e., SP-3d vs. Con-3d. The 3 d, 6 d and 9 d supplementation of spermine increased *claudin*-15 mRNA level, i.e., SP-3d vs. Con-3d, SP-6d vs. Con-6d, SP-9d vs. Con-9d. Moreover, the 3 d spermine administration enhanced *claudin*-15 mRNA levels, i.e., SP-3d vs. SP-7h ($P < 0.05$). The *MLCK* mRNA levels were decreased after 7 h and 3 d spermine supplementation, i.e., SP-7h vs. Con-7h; SP-3d vs. Con-3d.

3.3. Role of spermine on Rac1/PLC-γ1 signaling pathway in jejunum of piglets

Fig. 1A, B, C, D, E and F demonstrate the role of spermine on the mRNA levels of *Rac1*, *PLC-γ1*, *RhoA*, *CDC42*, *Rock1* and *MRLC* in the jejunum of piglets. Spermine treatment significantly increased the mRNA levels of *Rac1*, *PLC-γ1*, *RhoA*, *CDC42* and *MRLC* in jejunum ($P < 0.05$), but had no significant effect on *Rock1* gene expression ($P > 0.05$). The 6-d and 9-d spermine group significantly upregulated *Rac1* gene expression relative to 7-h spermine group ($P < 0.05$). Compared with the 3 d spermine group, the 6 d spermine group enhanced *Rac1* gene expression ($P < 0.05$). The 3-d spermine group enhanced the mRNA level of *PLC-γ1* relative to the 7-h control group ($P < 0.05$). Compared with the 7-h and 3-d spermine group, only 6-d spermine group significantly enhanced the mRNA levels of *RhoA*, *CDC42* and *MRLC* ($P < 0.05$). Prolonged time of spermine treatment had no significant effect on the expression of *Rock1* gene in jejunum ($P > 0.05$).

3.4. Correlation coefficient of Rac1/PLC-γ1 signaling pathway-related gene expression with barrier function-related mRNA levels in the jejunum and the content of D-lactate in serum

Table 3 shows that Rac1 mRNA level was positively related to gene expressions of *occludin*, *RhoA*, *CDC42* and *MRLC*. The *PLC-γ1* mRNA level was positively correlated with *claudin-15*, *claudin-14*, *claudin-3* and *ZO-2* gene expression levels. The level of *RhoA* gene expression was positively correlated with *claudin-12*, *claudin-1*, *CDC42* and *MRLC* mRNA levels. *CDC42* mRNA level was positively correlated with *occludin* and *MRLC* gene expression levels. The level of *MRLC* gene expression was negatively correlated with the content of serum D-lactate and positively correlated with *occludin* and *claudin*-1 mRNA levels in the jejunum.

3.5. Role of spermine on the expression of Rac1/PLC-γ1 signaling pathway-related mRNA levels in TNF-α-induced intestinal epithelial barrier injury model of IPEC-J2 cells

Fig. 2A, B, C, D, E and F show the role of spermine on the expression of Rac1/PLC-γ1 signaling pathway related-genes *Rac1*, *PLC-γ1*, *MRLC*, *RhoA*, *CDC42* and *Rock1* after TNF-α challenge. Compared with the control group, the 40 ng/mL TNF-α significantly decreased the *Rac1*, *RhoA* and *CDC42* gene expression ($P < 0.05$), whereas the 0.1 μmol/L spermine significantly increased the *Rac1*, *PLC-γ1*, *RhoA*, *CDC42* and *Rock1* gene expression levels ($P < 0.05$). The 40 ng/mL TNF-α + 0.1 μmol/L spermine group significantly increased the mRNA levels of *Rac1*, *PLC-γ1*, *MRLC*, *RhoA*, *CDC42* and *Rock1* relative to the 40 ng/mL TNF-α group ($P < 0.05$). Compared with the 40 ng/mL TNF-α + 0.1 μmol/L spermine group, the 40 ng/mL TNF-α + 0.1 μmol/L spermine +160 μmol/L NSC-23766 significantly decreased the *Rac1*, *MRLC*, *CDC42* and *Rock1* gene expression levels ($P < 0.05$), whereas the 40 ng/mL TNF-α + 0.1 μmol/L spermine + 3 μmol/L U73122 significantly decreased the *Rac1*, *PLC-γ1* and *MRLC* gene expression levels ($P < 0.05$).

Table 1
Roles of spermine administration on the content of D-lactate in the serum of piglets.[1]

Parameter	Treatment time								SD	*P*-value		
	7 h		3 d		6 d		9 d			SP	Time	SP × Time
	Con	SP	Con	SP	Con	SP	Con	SP				
D-lactate, μmol/L	61.62[a]	41.2[c]	42.54[c]	36.64[c]	57.09[ab]	40.9[c]	52.31[b]	39.25[c]	9.78	0.000	0.000	0.005

Con = control group; SP = spermine-supplemented group.
[a to c] Values with a different superscript differ were considered significant at $P < 0.05$.
[1] Values are means with their SD ($n = 6$, number of replicates).

Table 2
Roles of spermine on gene expressions of the jejunum barrier function in piglets.[1].

Parameters	Treatment time								SD	*P*-value		
	7 h		3 d		6 d		9 d			SP	Time	SP × Time
	Con	SP	Con	SP	Con	SP	Con	SP				
ZO-1	1.00^{b}	0.89^{b}	0.89^{b}	1.32^{a}	0.92^{b}	1.37^{a}	0.59^{c}	0.64^{c}	0.31	0.000	0.000	0.000
ZO-2	1.00^{ab}	1.09^{a}	0.94^{ab}	1.79^{ab}	1.01^{ab}	1.27^{a}	0.70^{b}	1.09^{ab}	0.34	0.000	0.001	0.008
Occludin	1.00^{c}	1.07^{c}	1.13^{bc}	1.36^{ab}	1.18^{bc}	1.62^{a}	0.86^{d}	1.09^{d}	0.33	0.002	0.003	0.508
Claudin-1	1.00^{abc}	0.95^{bc}	0.89^{bc}	1.30^{ab}	1.03^{abc}	1.45^{a}	0.83^{c}	1.14^{abc}	0.37	0.018	0.229	0.407
Claudin-2	1.00^{cd}	1.08^{c}	1.24^{abc}	1.38^{a}	1.09^{bc}	1.33^{ab}	0.56^{e}	0.78^{de}	0.31	0.004	0.000	0.699
Claudin-3	1.00^{b}	1.18^{b}	1.78^{a}	1.99^{a}	1.21^{b}	1.79^{a}	0.94^{b}	1.24^{b}	0.44	0.000	0.000	0.187
Claudin-12	1.00^{ab}	1.02^{ab}	0.96^{a}	1.30^{ab}	0.96^{a}	1.66^{ab}	0.68^{b}	1.02^{ab}	0.35	0.000	0.001	0.019
Claudin-14	1.00^{b}	1.53^{ab}	1.37^{b}	3.28^{a}	1.35^{b}	2.10^{ab}	1.23^{b}	1.63^{ab}	0.80	0.000	0.000	0.003
Claudin-15	1.00^{bc}	0.83^{d}	1.13^{b}	1.64^{a}	0.66^{e}	0.94^{cd}	0.50^{f}	0.88^{cd}	0.34	0.000	0.000	0.000
MLCK	1.00^{a}	0.58^{bc}	0.55^{bc}	0.32^{d}	0.48^{c}	0.62^{bc}	0.65^{bc}	0.45^{bcd}	0.19	0.000	0.000	0.000

Con = control group; SP = spermine-supplemented group; *ZO* = zonula occludens; *MLCK* = myosin light-chain kinase.
[a to f] Within a row, values with a different superscript were considered significant at $P < 0.05$.
[1] Values are means with their SD ($n = 6$, number of replicates).

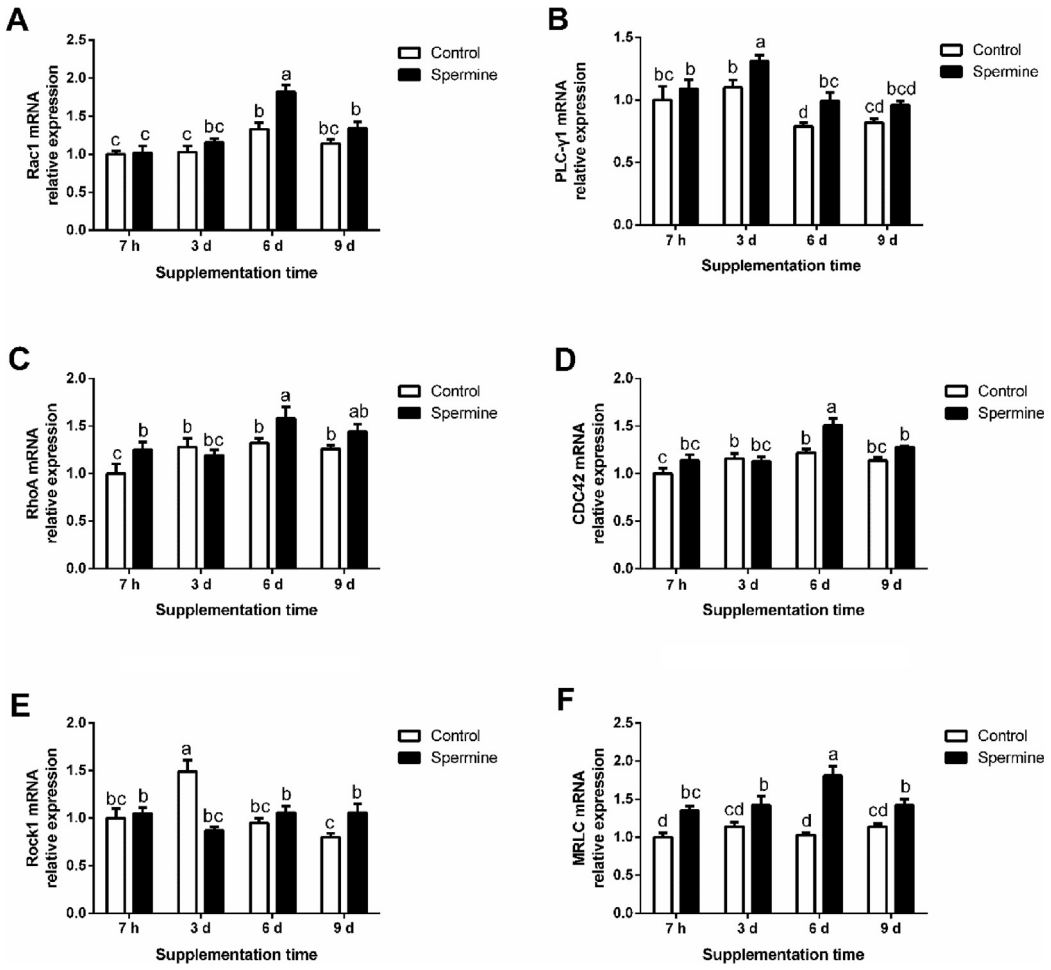

Fig. 1. Effects of spermine on the ras-related C3 botulinum toxin substrate 1 (Rac1)/phospholipase C-γ1 (PLC-γ1) signaling pathway mRNA levels in piglets' jejunum (A) *Rac1*, (B) *PLC-γ1*, (C) *RhoA*, (D) *CDC42*, (E) *Rock1* and (F) *MRLC*. Values are means with their SD ($n = 6$, number of replicates). Bars with a different superscript were considered significant at $P < 0.05$.

3.6. Effect of spermine on intestinal epithelial barrier function in injury model of IPEC-J2 cells induced by TNF-α

Table 4 demonstrates that spermine had a significant increase of TER and decrease of paracellular FD4 flux ($P < 0.05$); TNF-α had a decrease of TER and increase of FD4 flux relative to the control group ($P < 0.05$). Spermine supplementation decreased the TER drop and the paracellular dextran flux of FD4 enhancement induced by TNF-α ($P < 0.05$). However, NSC-23722 or U73122 blocked the protection roles of spermine in decreasing TER drop and paracellular permeability enhancement after TNF-α challenge.

Table 3
The correlation of intestinal barrier parameters, Rac1/PLC-γ1 signaling pathway in the jejunum of piglets.

Independent parameters	Dependent parameters	Correlation coefficients (r)[1]	P-value
Rac1	occludin	+0.762	0.028
	RhoA	+0.852	0.007
	CDC42	+0.946	0.000
	MRLC	+0.751	0.032
PLC-γ1	claudin-3	+0.708	0.049
	claudin-14	+0.721	0.044
	claudin-15	+0.926	0.001
	ZO-2	+0.775	0.024
RhoA	claudin-12	+0.721	0.043
	claudin-1	+0.765	0.027
	CDC42	+0.963	0.000
	MRLC	+0.783	0.037
CDC42	occludin	+0.731	0.039
	MRLC	+0.808	0.015
MRLC	occludin	+0.775	0.024
	claudin-1	+0.815	0.014
Diamine oxidase	claudin-1	−0.727	0.041
	occludin	−0.760	0.029
	Rac1	−0.836	0.010
	RhoA	−0.864	0.006
	CDC42	−0.876	0.004
	MRLC	−0.768	0.026

Rac1 = ras-related C3 botulinum toxin substrate 1; PLC-γ1 = phospholipase C-γ1; RhoA = ras homolog gene family member A; CDC42 = cell division control protein 42; MRLC = myosin regulatory light chain; ZO-2 = zonula occludens-2.
[1] r stands for Pearson correlation coefficient.

Fig. 3A, B, C and D show the effect of spermine on the protein concentration related to Rac1/PLC-γ1 signaling pathway in IPEC-J2 cells after TNF-α challenge. The 40 ng/mL TNF-α significantly reduced the occludin protein expression ($P < 0.05$) but had no significant effect on the expression of ZO-1 and claudin-1 proteins relative to the control group ($P > 0.05$). The 0.1 μmol/L spermine significantly enhanced the occludin and claudin-1 protein expression relative to the control group ($P < 0.05$). Compared with the 40 ng/mL TNF-α group, the 40 ng/mL TNF-α + 0.1 μmol/L spermine significantly increased the ZO-1, occludin and claudin-1 protein expression ($P < 0.05$). Compared with the 40 ng/mL TNF-α + 0.1 μmol/L spermine group, the 40 ng/mL TNF-α + 0.1 μmol/L spermine +160 μmol/L NSC-23766 group and 40 ng/mL TNF-α + 0.1 μmol/L spermine +3 μmol/L U73122 group reduced the ZO-1, occludin and claudin-1 proteins expression ($P < 0.05$).

3.7. Role of spermine on Rac1 and PLC-γ1 protein concentration in intestinal epithelial barrier injury model of IPEC-J2 cells after by TNF-α challenge

Fig. 4A, B, C, D and E show the role of spermine on the concentration of Rac1 and PLC-γ1 proteins in cells after TNF-α challenge. The 0.1 μmol/L spermine significantly increased the GTP-rac1 content and GTP-rac1/total Rac1($P < 0.05$). The 40 ng/mL TNF-α + 0.1 μmol/L spermine significantly increased the concentration of GTP-rac1 and PLC-γ1 phosphorylated proteins, as well as GTP-rac1/total Rac1 ratio relative to the 40 ng/mL TNF-α group ($P < 0.05$). The 40 ng/mL TNF-α + 0.1 μmol/L spermine +160 μmol/L NSC-23766 group and 40 ng/mL TNF-α + 0.1 μmol/L spermine +3 μmol/L U73122 group significantly decreased the concentration of GTP-rac1 and PLC-γ1 phosphorylated proteins, as well as GTP-rac1/total Rac1 ratio relative to the 40 ng/mL TNF-α + 0.1 μmol/L spermine group ($P < 0.05$).

4. Discussion

Our previous studies demonstrated that spermine supplementation can increase intestinal development and antioxidant status of piglets (Fang et al., 2016). We extended our current experiment into jejunal tissue to examine the effect of spermine on intestinal barrier integrity using the same animals. Spermine can induce precocious maturation of the intestine and has been shown to have beneficial effects in healing and prevention of gastrointestinal damage. The jejunum is the main segment of digestion and absorption of nutrients. The intestine is prone to damage when it experiences stress. The effect and mechanism of spermine on the intestinal (especially jejunal section) barrier integrity of piglets is largely unknown. Thus, the jejunal section was emphasized in this study. The intestinal barrier maintains intestinal immune response by affecting the integrity of the intestine (Rescigno, 2011). The intestine is mainly composed of the mucous layer in the intestinal mucosa, intestinal epithelial cells and their close connections and the underlying layers of the mucosa. Tight junctions are the most important intercellular connections. Various proteins constitute the tight junction, such as occludin, claudin, junction adhesion molecule and peripheral cytoplasmic proteins such as ZO-1 and ZO-2. Occludin is a transmembrane protein that maintains and regulates tight connections (Cummins, 2012). In this study, spermine intake enhanced the *occludin* mRNA levels in the jejunum of piglets. Claudin is the most important component of tight connections and mainly regulates the permeability of the intestinal barrier (Kinugasa et al., 2000). Our results showed that spermine ingestion increased the tight junction gene expression levels in the jejunum of piglets. Additionally, the occludin and actin skeleton can form a stable system by the C terminal of the peripheral membrane protein ZO-1 by combining with actin and stress fibre. ZO-2 interacts with ZO-1 to co-exist in the tight junction of epithelial cells and the adhesion site of non-epithelial cells (Itoh et al., 1999). Our study demonstrated that *ZO-1* and *ZO-2* gene expression levels were improved after spermine intake in the jejunum of piglets. ZO-1 distribution was affected by MLCK, which is a kind of calmodulin-dependent kinase causing the cytoskeleton to contract and damage the barrier function (Cunningham et al., 2012; Gallagher et al., 1997). This finding is in agreement with our result that spermine administration decreased the *MLCK* mRNA levels in the 7 h and 3 d supplementation in the jejunum of piglets. Serum D-lactate can reflect the degree of intestinal mucosal damage and permeability (Ewaschuk et al., 2005). Diamine oxidase reflects the degree of integrity and damage of the intestinal physical barrier. The results of our experiment showed that feeding spermine for 7 h, 3, 6 and 9 d significantly reduced the content of serum D-lactate. Fang et al. found that an extended spermine treatment time decreased the activity of diamine oxidase in the jejunum of piglets (Fang et al., 2016). In this study, the activity of diamine oxidase was negatively correlated with claudin-1 and occludin mRNA levels in the jejunum of piglets, respectively. Therefore, spermine supplementation may protect the intestinal integrity in vivo. This result was consistent with in the in vitro study, which found that spermine significantly increased TER and reduced paracellular permeability in IPEC-J2 cells. Occludin and claudin-1 proteins expression were significantly increased after 0.1 μmol/L spermine treatment. The results of this experiment are in agreement with the result of previous studies: the expression of occludin protein in IEC-Cdx2L1 cells was significantly decreased after depletion of polyamine treatment with DFMO (Yu et al., 2011). In this study, 0.1 μmol/L spermine significantly enhanced TER and the protein expression of

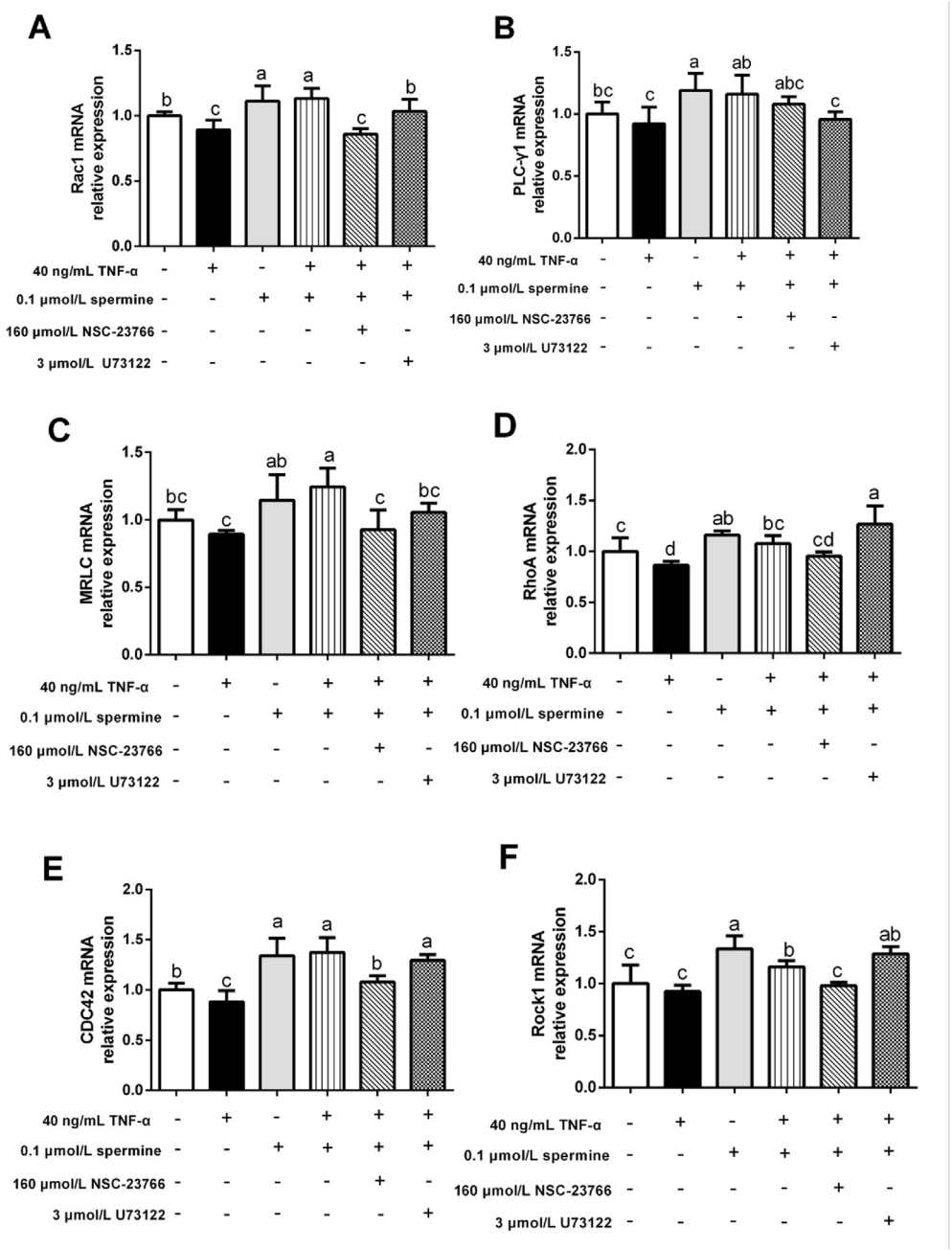

Fig. 2. Roles of spermine on the Rac1/PLC-γ1 signaling pathway-related gene expression in IPEC-J2 cells after TNF-α challenge. (A to F) represent mRNA relative expression of *Rac1*, *PLC-γ1*, *MRLC*, *RhoA*, *CDC42* and *Rock1*, respectively. Values are means with their SD ($n = 6$, number of replicates). [a to c] Bars with a different superscript were considered significant at $P < 0.05$.

Table 4
Intestinal epithelial barrier function.[1].

Item	Control	Spermine	TNF-α	Spermine + TNF-α	Spermine + TNF-α + NSC-23766	Spermine + TNF-α + U73122	SD	*P*-value
TER, %	100.00[b]	140.16[a]	44.96[d]	101.12[b]	57.60[c]	46.40[d]	4.27	0.00
FD4	1.00[e]	0.60[f]	1.31[c]	1.13[d]	1.84[a]	1.61[b]	0.03	0.00

TNF-α = tumor necrosis factor α; TER = transepithelial electrical resistance; FD4 = fluorescein-5-isothiocyanate dextran (4 kDa).
[a to f] Within a row, values with a different superscript were considered significant at $P < 0.05$.
[1] The values were normalized to control.

tight junction, and reduced paracellular permeability after TNF-α challenge, indicating that 0.1 μmol/L spermine could restore an injury to the barrier of porcine intestinal epithelial cells. This result was consistent with the findings of previous studies which found that arginine alleviated the damage of ZO-1 protein in IPEC-J2 cells induced by hypoxia (Chapman et al., 2012). Taken together,

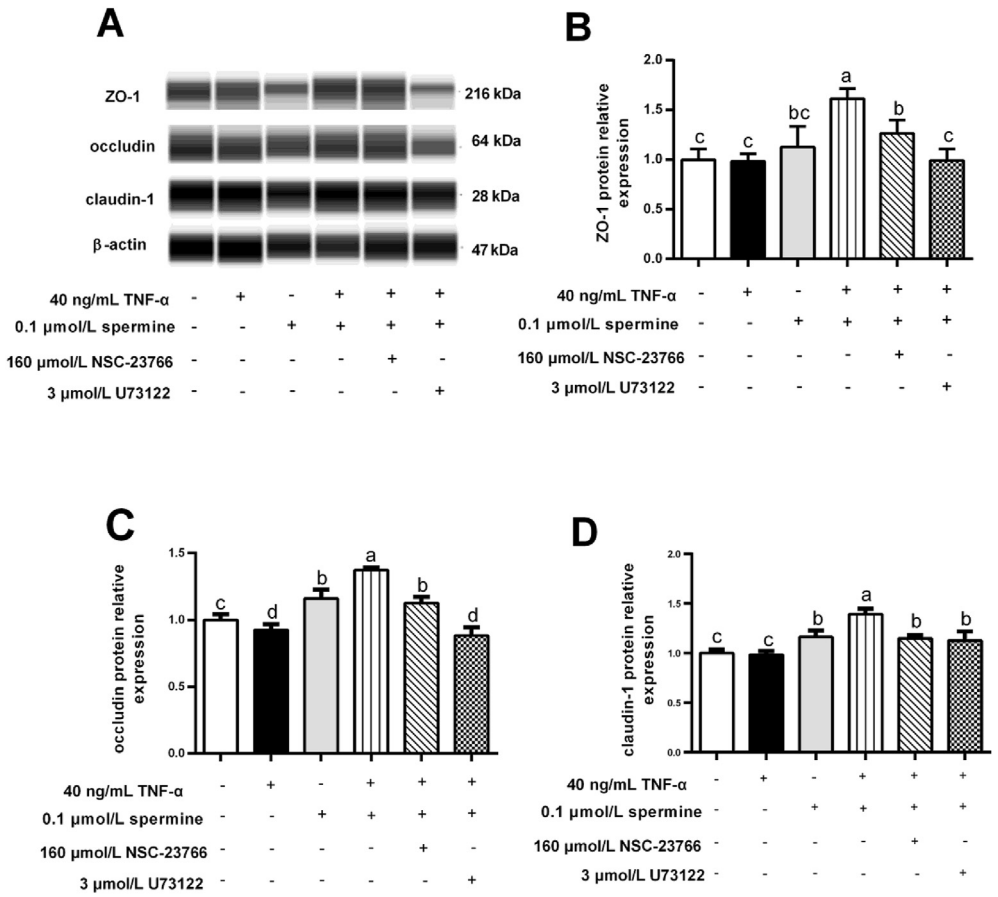

Fig. 3. Roles of spermine treatment on the expression of tight junction protein in IPEC-J2 cells after TNF-α challenge. Tight junction protein expression was detected by capillary Western blot. (A) Virtual blot view of Wes results showing ZO-1, occludin, claudin-1 and β-actin. (B to D) Relative protein expression of ZO-1, occludin and claudin-1, respectively. Values are means with their SD ($n = 6$, number of replicates). [a to d] Bars with a different superscript were considered significant at $P < 0.05$.

spermine protects intestinal integrity by improving the TER and expression of tight junction protein, and decreasing paracellular permeability.

The *RhoA*, *Rac1*, *PLC-γ1* and *CDC42* gene expression levels are closely related to the protein expression of tight junction (Bruewer et al., 2004; Hirase et al., 2001; Jou et al., 1998; Klein, 2007). In our experiment, the *Rac1* gene expression was positively correlated with the mRNA levels of *occludin*. *PLC-γ1* gene expression was positively correlated with the tight junction mRNA levels in the jejunum. In addition, a positive correlation was observed between *RhoA* and *claudin-1*, *claudin*-12 mRNA levels, respectively. Moreover, the mRNA levels of *occludin* were positively related to the *CDC42* gene expression. The gene expression of *MRLC* was also positively related to the mRNA levels of *occludin* ($r = +0.775$, $P = 0.024$) and *claudin-1* ($r = +0.815$, $P = 0.014$). These results suggested that the improvement of tight junction mRNA levels may be partly ascribed to the upregulation of the mRNA levels of Rac1/PLC-γ1 signaling pathway in the jejunum of piglets. However, whether the Rac1/PLC-γ1 signaling pathway is involved or not in the restoration process is unclear. Therefore, we explored the relationship between spermine and the Rac1/PLC-γ1 signaling pathway.

Rac1 controls glucose uptake, cell growth, cytoskeleton recombination and protein kinase activation (Sun et al., 2004). Our findings also showed that spermine treatment significantly enhanced the expression of *Rac1*, *PLC-γ1*, *RhoA*, *CDC42* and *MRLC* genes in jejunum, indicating that spermine may restore the intestinal epithelium of piglets possibly by regulating the gene expression related to Rac1/PLC-γ1 signaling pathway. Moreover, CDC42 is the downstream gene of the activated Rac1 in primary keratinocytes from mice (Du et al., 2009). In this study, a significant positive correlation between the Rac1 and CDC42 gene expression levels in the jejunum suggested that the increased *CDC42* mRNA levels were partly ascribed to the improved *Rac1* gene expression. In the in vitro study, spermine significantly increased the expression of *Rac1*, *PLC-γ1*, *RhoA*, *CDC42*, *Rock1* and MRLC genes in cells. Spermine can also significantly increase the expression of *Rac1*, *PLC-γ1*, *RhoA*, *CDC42* and *Rock1* genes in the intestinal epithelial barrier injury model of IPEC-J2 cells induced by TNF-α. These findings were consistent with previous studies indicating that spermidine increases the gene expression levels of *Rac1*, *PLC-γ1*, *RhoA* and *CDC42* in IEC-6 cells (Song et al., 2015, 2016) and asymmetric dimethylarginine increases the expression level of Rock1 gene in rat pulmonary artery smooth muscle cells (Liu et al., 2015). In this study, spermine significantly enhanced GTP-rac1 protein content and GTP-rac1/total rac1 ratio in cells. This result was in agreement with a previous study indicating that Rac1 protein expression is reduced by putrescine depletion in IEC-Cdx2L1 cells (Cummins, 2012) and DFMO treatment in differentiated IEC-Cdx2L1 cells (Rao et al., 2008). Moreover, putrescine depletion decreased the expression level of GTP-rac1 protein in IEC-6 cells (Ray et al., 2003, 2007). Further, our experiment showed that 40 ng/mL TNF-α + 0.1 μmol/L spermine +160 μmol/L NSC-23766 significantly decreased TER and protein expression of tight junction, and the content of GTP-rac1

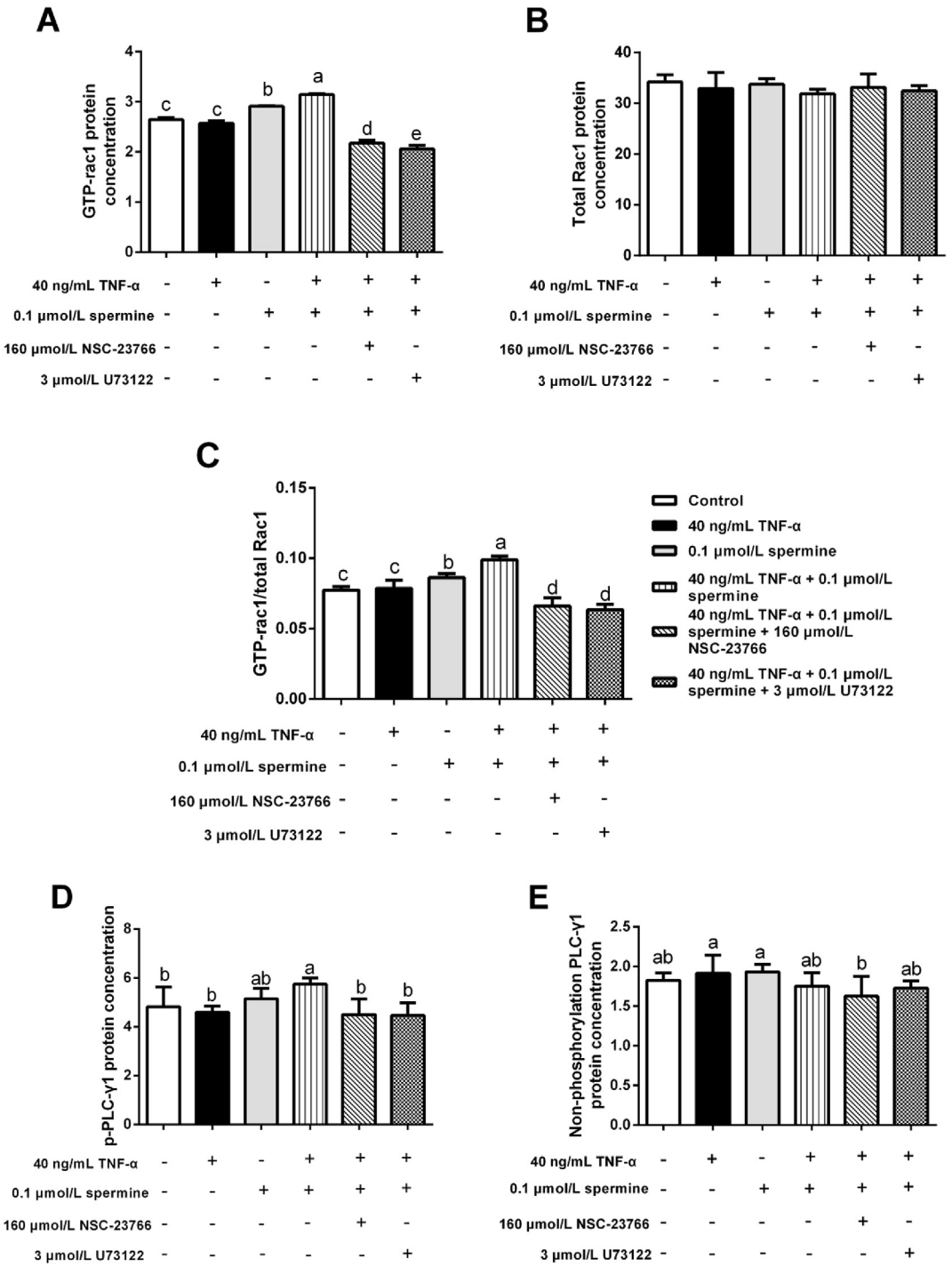

Fig. 4. Roles of spermine on Rac1 and PLC-γ1 protein concentration in IPEC-J2 cells after TNF-α challenge. (A to E) represent for GTP-rac1, total Rac1, GTP-rac1/total Rac1, p-PLC-γ1 and non-phosphorylation PLC-γ1, respectively. Values are means with their SD ($n = 6$, number of replicates). [a to e] Bars with a different superscript were considered significant at $P < 0.05$.

and PLC-γ1 phosphorylated proteins, as well as the gene expression of *Rac1*, *MRLC*, *CDC42* and *Rock1*, and increased paracellular permeability in the presence of 40 ng/mL TNF-α and 0.1 μmol/L spermine. The 40 ng/mL TNF-α + 0.1 μmol/L spermine +3 μmol/L U73122 significantly decreased TER and the expression of ZO-1, occludin, claudin-1, GTP-rac1, PLC-γ1 phosphorylated protein and *Rac1*, *PLC-γ1*, *MRLC* genes and increased paracellular permeability, suggesting that spermine protects intestinal barrier integrity through the Rac1/PLC-γ1 signaling pathway in pigs. Results of our experiment are in agreement with findings of the previous study: with the prolongation of the treatment time of Rho kinase inhibitor Y27632, the expression of GTP-rac1, GTP-RhoA protein decreased significantly in scratch wound IEC-6 cells (Bavaria et al., 2011).

5. Conclusion

Spermine increases the levels of tight junction protein expression and trans-epithelial electrical resistance, and decreases

paracellular permeability, thus promotes intestinal barrier integrity. Spermine protects intestinal barrier integrity through the Rac1/PLC-γ1 signaling pathway in pigs. This study is the first to explore the mechanism of spermine supplementation modulating intestinal integrity of pigs.

Author contributions

Guangmang Liu conceived, designed, conducted the experiment, wrote the paper and had primary responsibility for the final content. **Xiaomei Xu** conceived, designed, conducted the experiment and wrote the paper. **Caimei Wu, Gang Jia, Hua Zhao, Xiaoling Chen, Gang Tian, Jingyi Cai** and **Jing Wang** conducted the methodology and validation.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Supplementary data

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