

# Niacin Alleviates the Acidosis of Rumen Epithelial Cells Induced by Low pH Resulting from Volatile Fatty Acids

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## ABSTRACT

To investigate the effects of different niacin treatments on low pH-induced goat rumen epithelial cells acidosis, cells were exposed to solutions with a pH of 7.4, 5.8, 5.6, 5.2, and 5.0 for 3, 6, 12, and 24 h. The precise pH was adjusted using volatile fatty acids (acetate, propionate, and butyrate). All the cell viability in low pH treatment declined more than 50% for 12 h stimulation, and cells treated with low pH for 12 h was selected to be optimal ruminal acidosis model. Cells were then cultured with niacin (0, 20, 40, 80, 160, or 320 mM) for 12 h before, concurrent, and after 12 h treatment with low pH. Results showed that, 1) Pre-treatment protection of niacin: pre-treatment with 20 or 40 mM niacin increased the cell viability under pH 5.8, while 80-320 mM niacin decreased the cell viability compared with the control group ( $P < 0.01$ ). Under pH 5.5, results were similar to those under pH 5.8 except for pre-treatment with 40 mM niacin with no significant effect on the cell viability. Under pH 5.2 or 5.0, pre-treatment with niacin reduced the cell viability compared with the control group ( $P < 0.01$ ). 2) Concurrent treatment protection of niacin: under all pH value, all concurrent treatment with niacin reduced the cell viability compared with the control group ( $P < 0.01$ ). 3) Post-treatment protection of niacin: under pH 5.8 or 5.5, post-treatment with 20 or 40 mM niacin increased the cell viability compared with the control group, while 80-320 mM niacin decreased the cell viability ( $P < 0.01$ ). Under pH 5.2 or 5.0, post-treatment with niacin reduced the cell viability compared with the control group ( $P < 0.01$ ). These results may provide a reference for ruminal acidosis mitigation. Low-concentration niacin could relieve subacute ruminal acidosis, and it could be added after symptoms appear. However, niacin had a negative role in the protection of acute ruminal acidosis.

## INTRODUCTION

In modern ruminant's production system, high-concentrate in the diet is widely used to improve production performance and economic benefits. Nevertheless, the overfeeding of a high-concentrate diet leads to an accumulation of volatile fatty acids (VFA), which exceed

VFA absorption of rumen epithelium (Li *et al.*, 2019), thus leads to lower rumen pH and finally results in rumen acidosis (Steele *et al.*, 2012; Sun *et al.*, 2018). The prolonged low pH condition promotes the cytolysis of gram-negative bacteria and releases lipopolysaccharides (LPS). Meanwhile, ruminal bacteria can decarboxylate histidine to produce histamine under excessively low acidic environment. The LPS and histamine disrupt the integrity of the ruminal epithelial structure and further damages barrier functions of rumen mucosa (Plaizier *et al.*, 2012; Mao *et al.*, 2015). When damage in the ruminal epithelial structure reaches a certain level, abnormal metabolites and pathogenic microorganisms migrate into the blood through the damaged rumen mucosa, further lead to immunosuppression and inflammatory response on the animals (Khafipour *et al.*, 2009; Plaizier *et al.*, 2012; Dong *et al.*, 2013). This can induce a series of diseases, such as

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## Authors' Contribution

Conceptualization, MS, QQ and KO; Methodology, MS and YL; Validation, YL, XZ and LX; Formal analysis, MS and YL; Investigation, MS, YL, HL, KP and MQ; Data curation, MS, ZG and YL; Writing original draft preparation, MS; Writing review and editing, YL, QQ and KO; Visualization, YL, QQ and KO; Supervision, QQ and KO; Project administration, QQ and KO; Funding acquisition, KO.

## Key words

Low pH, Niacin, Rumen epithelial cell, Ruminal acidosis, Volatile fatty acids

diarrhoea, rumenitis, laminitis, and liver abscesses, which ultimately affect the health and production performance of ruminant (Nagaraja and Titgemeyer, 2007; Plaizier *et al.*, 2012). Therefore, inhibiting low pH-induced rumen mucosal damage with maintaining the integrity of the ruminal epithelial cells is a formidable challenge that may improve ruminant health and production performance.

Niacin is a precursor for the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) *in vivo*, participating in various hydrogen transfer processes. Therefore, gastrointestinal epithelium with a high cell turnover rate requires more niacin (Kennedy, 2016). In addition, niacin is also involved in mitochondrial and DNA respiration, cell signaling transduction, intracellular poly ADP-ribose polymerase (PARP) production (Khafipour *et al.*, 2009; Surjana *et al.*, 2010). Therefore, niacin is an important protective vitamin in cells.

Our previous study found that adding an appropriate concentration (20–80mM) of niacin can attenuate butyrate-induced damage and promote the proliferation of rumen epithelial cells *in vitro* (Luo *et al.*, 2019). However, to the best of our knowledge, the effect of niacin on rumen epithelial cell damage induced by low-pH with VFA *in vitro* has not been reported. The objectives of this study were to investigate the protective effects of niacin supplementation on rumen epithelial cells acidosis induced by low-pH. These results of this study may provide a reference for the mitigation of ruminal acidosis induced by low-pH with VFA accumulation.

## MATERIALS AND METHODS

### *Isolation and culture of rumen epithelial cells*

Rumen epithelial cells were isolated from the rumen epithelial tissues of 8-month-old male Boer goats with similar body weight ( $45\pm5$ kg). Goats were sacrificed via electrical stunning and followed by exsanguination. Immediately after death, the abdominal cavity was quickly opened, and the rumen was removed. A 5cm × 5cm rumen tissue was taken from the caudoventral blind sac, repeatedly washed with sterile to remove the feed impurity. Then the rumen samples were put into a culture medium bottle containing 10% fetal bovine serum and brought back to the laboratory.

The following steps were performed in biohazard safety equipment. Briefly, the rumen sample was vortexed five times for 10 s by ice-cold antibiotic-free PBS (Vortex Mixer, Fisher Scientific). The rumen epithelium was then peeled away from the connective tissue, vortexed five times for 10 sec by ice-cold PBS with 0.5 mg/mL amphotericin B and 100 µg/mL gentamicin, cut into small pieces (about 1 mm<sup>3</sup>), and washed with D-Hank's buffer until the buffer

remained clear. Subsequently, the rumen epithelial cells were isolated from the mucosae using 0.1% collagenase I and 0.25% trypsin-0.02% EDTA. Then, the rumen epithelial cells were seeded sequentially at a density of  $1\times10^6$  cell/mL and cultured in DMEM supplemented with 10% (v/v) FBS, 0.5% mEGF and 1% (v/v) streptomycin/amphotericin B in 25 cm<sup>2</sup> plastic cell culture flasks at 37°C and 5% CO<sub>2</sub> in an incubator (Thermo Fisher Scientific, Rockford, USA). The medium was replaced every 24 h, and the pH was maintained at 7.4. When the cells reached 70% confluence, they were trypsinized (0.25% trypsin-0.02% EDTA in PBS) and subcultured at  $3\times10^5$  cells/25 cm<sup>2</sup> in culture flasks at 37 °C with 5% CO<sub>2</sub>. In this study, cells from passages 1–3 were used. No immortal rumen epithelial cell line is available.

### *Cell treatment*

Rumen epithelial cells were inoculated in 96-well plates in the appropriate medium. When it reached 80% confluence, cells were treated with different solutions with pH of 7.4, 5.8, 5.6, 5.2, or 5.0 for 3, 6, 12, or 24 h in 96-well plates with six repetitions per group to determine the effects of different solution pH and duration time on cell activity. The solution was a mixture consisting acetate, propionate, and butyrate with a volume ratio of 6:3:1, and sodium hydroxide was used for the precise pH adjustment. Due to all the cell viability in low pH treatment declined more than 50% for 12-h stimulation, cells treated with low pH for 12 h were determined to be optimal ruminal acidosis model *in vitro*. Cells were then cultured with niacin (0, 20, 40, 80, 160, or 320 mM) for 12 h before, concurrent, and after 12 h treatment with low pH (5.8, 5.6, 5.2 or 5.0) in 96-well plates with six repetitions per group, to determine the effects of different niacin treatment on cell viability under low-pH.

### *Cell viability assay*

Cell viability was determined by MTT assay according to the method of Mosmann (1983). Briefly, rumen epithelial cells were inoculated in 96-well plates at a density of  $5\times10^3$  cells/well. After cells treated as mentioned above, MTT solution (5 mg/mL in PBS) was added to each well, and then the cells were incubated for 4 h. The medium was removed, and 150 µL DMSO/well was added. The Formazan crystals inside the cells were dissolved by placing the plates on a vibrator for 10 minutes. The absorbance was measured at 570 nm using a microplate spectrophotometer (Thermo Fisher Scientific, Rockford, USA). Relative cell viability was presented as a percentage relative to the control group. All independent experiments were performed in triplicate.

### *Statistical analysis*

All results are presented as the mean±standard

deviation (SD). Statistical Package for the Social Sciences (SPSS 22.0, IBM Corporation, Armonk, New York, United States) packages were used for statistical analyses. Differences among groups were tested using a one-way analysis of variance (ANOVA). Duncan's multiple comparison test was used to compare statistical differences between treatments. A value of  $P < 0.05$  was considered to be significant.

## RESULTS

### Effects of pH and duration time on cell activity

For 3-h low pH stimulation, the cell viability was significantly decreased in the pH 5.5, 5.2, or 5.0 group as compared to the normal pH (pH 7.4), and dropped by about more than 50% ( $P < 0.01$ ). In contrast, the cell viability was not affected significantly by the pH 5.8 (Fig. 1a). For 6-h low pH stimulation, compared to the normal pH (pH 7.4), the cell viability was significantly decreased in the pH 5.8, 5.5, 5.2, or 5.0 group, but did not drop by about 50% ( $P < 0.01$ ) (Fig. 1b). For 12-h low pH stimulation, compared to the normal pH (pH 7.4), the cell viability was significantly decreased in the pH 5.8, 5.5, 5.2, or 5.0 group, and dropped by more than 50% ( $P < 0.01$ ) (Fig. 1c). For 24-h low pH stimulation, compared to the normal pH (pH 7.4), the cell viability was significantly decreased in the pH 5.8, 5.5, 5.2, or 5.0 group, the cell viability did not drop by about 50% in the pH 5.8 group, while the cell viability dropped by almost more than 50% in the pH 5.5, 5.2, or 5.0 group ( $P < 0.01$ ) (Fig. 1d). Due to all the cell viability in low pH treatment declined more than 50% for 12-h stimulation, cells treated with low pH for 12 h were determined to be optimal ruminal acidosis model *in vitro*.

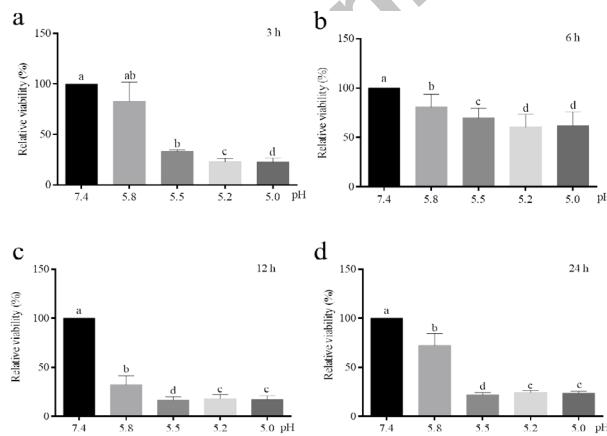


Fig. 1. Effects of different solution pH and duration time on cell activity. Cells were treated with different solutions with pH of 7.4, 5.8, 5.6, 5.2 or 5.0 for 3, 6, 12 or 24 h. Relative cell viability was presented as a percentage relative to the control group. <sup>a,b,c</sup> Mean values within different letters were significantly different ( $P < 0.05$ ).

### Effect of niacin on cell viability under low pH

Under pH 5.8 or 5.5, compared to the untreated control, pre-treatment with 20 or 40 mM niacin significantly increased the cell viability, while pre-treatment with 80, 160, or 320 mM niacin significantly decreased the cell viability ( $P < 0.01$ ) (Fig. 2a, 2b). Under pH 5.2 or 5.0, all pre-treatment with niacin (20–320 mM) significantly reduced the cell viability compared with the control group ( $P < 0.01$ ) (Fig. 2c, 2d).

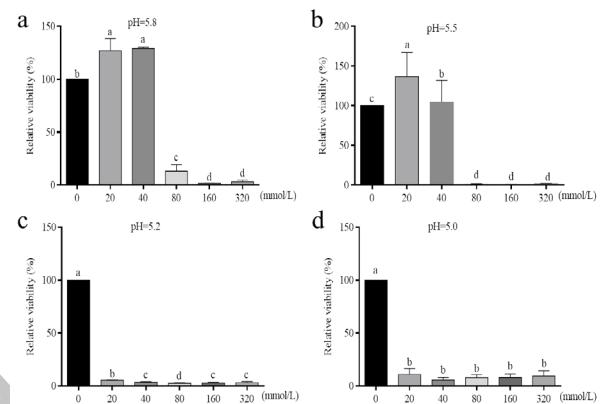


Fig. 2. Effects of pre-treatment of niacin on cell viability under low pH. Cells were cultured with niacin (0, 20, 40, 80, 160, or 320 mM) for 12 h before 12 h treatment with low pH (5.8, 5.6, 5.2 or 5.0). Relative cell viability was presented as a percentage relative to the control group. <sup>a,b,c</sup> Mean values within different letters were significantly different ( $P < 0.05$ ).

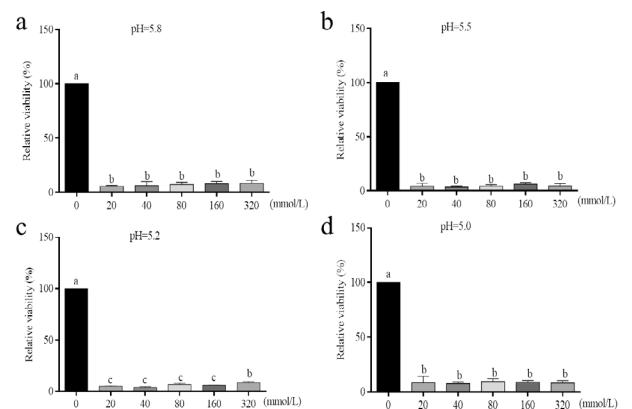


Fig. 3. Effects of concurrent treatment of niacin on cell viability under low pH. Cells were cocultured with niacin (0, 20, 40, 80, 160, or 320 mM) and low pH (5.8, 5.6, 5.2 or 5.0) for 12 h. Relative cell viability was presented as a percentage relative to the control group. <sup>a,b,c</sup> Mean values within different letters were significantly different ( $P < 0.05$ ).

### Concurrent treatment protection of niacin on cell viability under low pH

Under all pH value (5.8–5.0), compared to the untreated control, all concurrent treatment with niacin (20–320 mM) significantly reduced the cell viability compared with the control group ( $P < 0.01$ ) (Fig. 3).

### Post-treatment protection of niacin on cell viability under low pH

Under pH 5.8 or 5.5, compared to the untreated control, post-treatment with 20 or 40 mM niacin significantly increased the cell viability. In contrast, post-treatment with 80, 160, or 320 mM niacin significantly decreased the cell viability ( $P < 0.01$ ) (Fig. 4a, 4b). Under pH 5.2 or 5.0, all post-treatment with niacin (20–320 mM) significantly reduced the cell viability compared with the control group ( $P < 0.01$ ) (Fig. 4c, 4d).

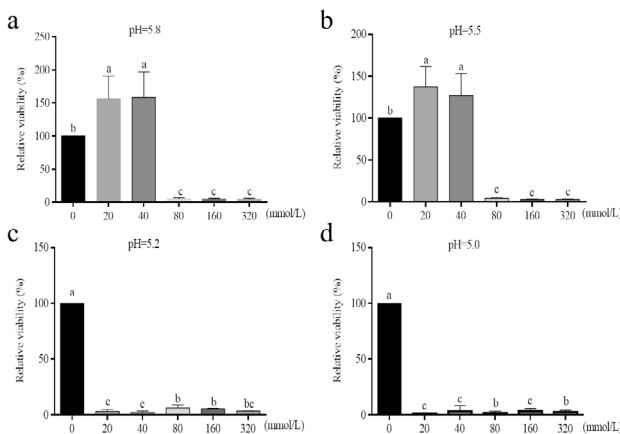


Fig. 4. Effects of post-treatment of niacin on cell viability under low pH. Cells were cultured with niacin (0, 20, 40, 80, 160, or 320 mM) for 12 h after 12 h treatment with low pH (5.8, 5.6, 5.2 or 5.0). Relative cell viability was presented as a percentage relative to the control group. <sup>a,b,c</sup> Mean values within different letters were significantly different ( $P < 0.05$ ).

### Pre-treatment protection vs. Post-treatment protection of niacin

Under pH 5.8 or 5.5, it can be seen that post-treatment with 20 or 40 mM niacin had numerically higher relative viability than pre-treatment with 20 or 40 mM, respectively (Fig. 5).

## DISCUSSION

The mechanisms of ruminal acidosis have been extensively investigated, three doctrines about the occurrence of ruminal acidosis were put forward, including

“lactic acidosis” (Enemark *et al.*, 2002), “organic acids (are mainly VFA and lactate) acidosis” (Krause and Oetzel, 2005), and “histamine and endotoxin poisoning” (Duffield *et al.*, 2004). Our previous study *in vitro* showed that niacin could alleviate the decline of rumen epithelial cell activity induced by lactic acid. However, there is little knowledge of the niacin protection for low pH-induced rumen acidosis caused by VFA *in vitro*. Therefore, this study investigated the niacin protection effects on cell activity of rumen epithelial cells in a goat rumen epithelial cells model using VFA accumulation simulations. In the present study, pH 5.8–5.0 were selected to simulate ruminal acidosis systems, due to that a pH threshold of 5.5 was used to define subacute ruminal acidosis (SARA) and pH below 5.2 for acute ruminal acidosis (ARA) (Penner *et al.*, 2007; Aschenbach *et al.*, 2011). The natural balance of acid-base is essential for the proper function of cells. A drop of extra- and intra-cellular pH is associated with a depression of cytosolic and membrane enzyme activity, ion transport, and protein and DNA biosynthesis (Busa and Nuccitelli, 1984). Consistently, according to the results, we found that the lower the pH value caused by VFA, the greater the cell damage was and the lower the cell activity was. This might confirm that the VFA accumulation and rumen pH decline induced by high-concentrate diet fermentation can directly damage the rumen epithelial cells in ruminants (Meissner *et al.*, 2017; Penner *et al.*, 2009).

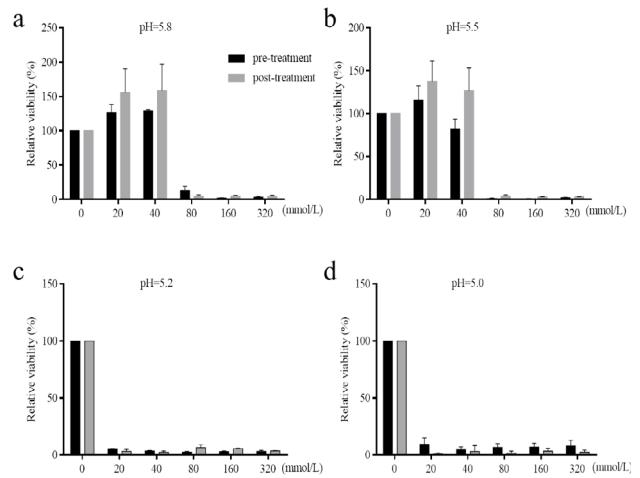


Fig. 5. Pre-treatment protection vs Post-treatment protection of niacin on cell viability under low pH.

In the present study, at low pH (5.5, 5.2, 5.0) stimulation time was prolonged, the cell activity of rumen epithelial cells decreased first (3 h) and then rose (6 h) and finally declined (12 h, 24 h) (Fig. 1). These results could be related to the ability of cells to adapt acid-base balance

(3–6 h), but the continuous mixed VFA stimulation could cause irreversible cell damage, ultimately leading to cell death (Meissner *et al.*, 2017; Pan *et al.*, 2007). Furthermore, VFA can promote the rapid development of the papilla and lead to rumen epithelial keratinization, further results in the damage of the rumen barrier (Kleen *et al.*, 2003; Steele *et al.*, 2011; Zhang *et al.*, 2013), the destruction of rumen tight junction proteins, and the acceleration of cell apoptosis (Heller *et al.*, 2005; Zeissig *et al.*, 2007). Therefore, cells treated with low pH for 12 h were determined to be optimal ruminal acidosis model *in vitro*.

Niacin is a precursor for the coenzymes NAD and NADP *in vivo*, which participate in DNA and mitochondrial respiration and have redox functions. Previous studies have reported that adding an appropriate concentration (40 mM) of niacin can inhibit butyrate-induced apoptosis of rumen epithelial cells *in vitro* by reducing intracellular oxidative stress, inhibiting the activation of caspase-3 and p53, and repairing DNA damage (Luo *et al.*, 2019). Consistently, in this study, under the pH value of SARA, pre-treatment or post-treatment low niacin content (20–40 mM) had a positive effect on the reduced cell viability at SARA pH. However, high niacin content (80–320 mM) decreased the cell viability, which means that niacin exhibits cytotoxic effects at high concentrations. We speculated that this might be associated with the interaction between niacin and PARP. PARP is a family of NAD-dependent enzymes, can be activated by DNA damage. They use NAD as substrate, promote the formation of poly (ADP-ribose) polymers (PAR) on substrate proteins (Amé *et al.*, 2004), regulating various physiological processes such as to maintenance genomic integrity (Jagtap and Szabo, 2005). Niacin prevents DNA damage through two possible pathways that involve PARP. First, niacin may prevent PARP degradation and allow for DNA repair through the direct inhibition of CPP32 activity (Lin *et al.*, 2000). Second, niacin supplementation leading to increased NAD allows cells to recover more efficiently after DNA damage (Jacobson *et al.*, 1999). However, excessive activation of PARP uses NAD to make a large amount of (ADP ribose) polymers, the regeneration of NAD from nicotinamide cause ATP depletion and subsequently lead to cell death because of metabolic derangement (Endres *et al.*, 1997). Therefore, excessive ATP-consuming reactions during DNA repair induced by high niacin concentration may explain its cytotoxic effects. Moreover, for ARA pH, rather than reversing rumen epithelial cells damage caused by mixed VFA, niacin treatment critically aggravate cell damage. This may also due to low pH (5.2 or 5.0) induced DNA damage indeed, lead to excessive PARP activation, cause ATP depletion, and subsequently lead to cell death in

the presence of niacin.

Interestingly, our results showed that no matter SARA pH or ARA pH, concurrent treatment with niacin (20–320 mM) all reduced the cell viability of rumen epithelial cells compared with the control group. In the present study, the concurrent treatment was to mix the niacin with mixed acid. While in the presence of niacin and at low pH, it can format useful quantities of nicotinic acid adenine dinucleotide phosphate (NAADP) (Asfaha *et al.*, 2019). NAADP is a potent Ca<sup>2+</sup> mobilizing second messenger that triggers Ca<sup>2+</sup> release in mammalian cells (Lee, 2005). The elevation of intracellular Ca<sup>2+</sup> leads to apoptosis of cells through synchronizing the mass exodus of cytochrome c from the mitochondria (Mattson and Chan, 2003). Therefore, the production of NAADP in the presence of niacin and mixed acid may aggravate cell apoptosis and decreased cell viability.

## CONCLUSION

In conclusion, pre-treatment or post-treatment of low-concentration niacin (20–40 mM) relieved rumen epithelial cell damage induced by low-pH, whereas high-concentration niacin exacerbated the cell damage. The treatment of niacin had no protective effect on cell damage at ARA pH. In addition, concurrent supplementation of niacin at low pH aggravates cell damage. These results may provide a reference for ruminal acidosis mitigation. A low-concentration niacin could relieve SARA, and it could be added after SARA symptoms appear, but not the responses to ARA.

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### *Statement of conflict of interest*

The authors have declared no conflict of interest.

### *Animal welfare statement*

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes. This study was

approved by the Animal Care and Use Committee of the College of Animal Science and Technology of Jiangxi Agricultural University (JXAULL-2020-27).

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