**Involvement of P38 and ERK1/2 in Mitochondrial Pathways Independent Cell
Apoptosis in Oviduct Magnum Epithelial Cells of Layers Challenged with
Vanadium**

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ABSTRACT

Vanadium (V) can induce cell apoptosis in layers' oviduct resulting in egg quality reduction. In this study, we investigated the relationship between the mitogen-activated protein kinase (MAPK)-signaling pathway and V-induced apoptosis in poultry oviduct magnum epithelial cells (OMECs). Cultured OMECs were divided into eight treatment groups: 0 $\mu\text{mol/L}$ V (control), 100 $\mu\text{mol/L}$ V (V100), V100+P38MAPK inhibitor (SB203580), SB203580, V100+extracellular signal-regulated kinases 1 and 2 (ERK1/2) inhibitor (U0126), U0126, V100+c-JUN NH₂-terminal kinase (JNK) inhibitor (SP600125), and SP600125. The OMECs were pretreated with the MAPK inhibitors before their treatment with V100 for 12 h. V100 increased the apoptosis of OMECs ($P<0.05$), while three MAPK inhibitors suppressed V100-induced apoptosis ($P<0.05$); V100 enhanced the depolarization of $\Delta\psi_m$ ($P<0.05$), and SB203580 and U0126 alleviated the V100-induced $\Delta\psi_m$ decrease ($P<0.05$); V100 downregulated B-cell lymphoma-2 (Bcl-2) and poly ADP-ribose polymerase 1 (PARP1) mRNA expression ($P<0.05$), meanwhile it

upregulated Bcl-2 associated x (Bax), Apaf1, cytochrome C (CytC) and cysteine aspartase (caspase) 3, 8, 9 mRNA expression ($P<0.05$). All MAPKs inhibitors alleviated the up-regulation of V100 for Bax and caspase 3 mRNA expression and down-regulation of V100 for Bcl-2 expression ($P<0.05$). SB203580 and U0126 upregulated CytC expression treated by V100 ($P<0.05$), except SP600125, while SB203580 administration resulted in a similar upregulation of PARP1 expression ($P<0.05$). SP600125 can alleviate V triggered p-P38MAPK (phosphor-P38), p-ERK1/2 (phosphor-ERK1/2), p-JNK (phosphor-JNK) increase on OME cells, and SB203580 and U0126 had a similar response to phosphor-P38 and p-JNK ($P<0.05$). It concluded that V induced apoptosis in OMECs through the activation of P38 and ERK1/2, and by increasing the ratio of Bax/Bcl-2, which resulted in $\Delta\psi_m$ decrease, CytC release into the cytosol; consequently caspase 3 is recruited and activated, PARP1 is cleaved, eventually leading to apoptosis.

Key words: mitogen-activated protein kinase, vanadium, apoptosis signaling pathway, oviduct magnum epithelial cells, mitochondrial membrane potential

1. Introduction

Vanadium (V) is a necessary trace element for carbohydrate and lipids metabolism of carbohydrate and lipids, thus sustaining animal health and growth [1]. However, the intake of excessive V has some toxic effects on animals, especially on poultry, which seems to be more vulnerable than other species [2]. Supplementation of more than 10 mg/kg V in layers' diet adversely affects egg quality, especially albumen height and Haugh units, which depend on the ovomucin levels in the albumen [3-5].

Albumen proteins were mainly synthesized and secreted by the oviduct magnum epithelial cells (OMECs), located in the interior walls of the magnum tract [6]. In our previous studies, vanadium (V^{5+}) not only induced apoptosis in the duodenal, magnum, and uterine epithelial cells of laying hens *in vivo* [7, 8], but also induced apoptosis in the OMECs of layers *in vitro* [9].

Apoptosis, known as a programmed cell death, is a fundamental event involved in the normal proliferation and differentiation of multicellular organisms [10]. The two types of apoptosis include an extrinsic pathway that involves transmembrane death receptor-mediated interactions, such as the death receptor Fas and its ligand FasL [11], and an intrinsic pathway that involves mitochondrial dysfunction, which is mediated by phosphorylated mitogen-activated protein kinases (MAPKs) to regulate the proportions of pro- and anti-apoptotic proteins, such as B-cell

lymphoma-2 (Bcl-2) and Bcl-2 associated x (Bcl-2/Bax). Higher pro-apoptotic protein expression and lower anti-apoptotic proteins expression can trigger changes in mitochondrial membrane potential ($\Delta\psi_m$), resulting in the release of cytochrome C (CytC) into the cytosol, which then binds to the apoptotic protease activating factor-1 (Apaf-1), causing a cysteine aspartase (caspase) family cascade reaction, cleaving poly ADP-ribose polymerase 1 (PARP1) and finally leading to apoptosis [12-14].

The MAPK family is a group of highly conserved, proline-directed serine/threonine kinases and includes three prominent members: P38MAPK (P38), extracellular signal-regulated kinases 1 and 2 (ERK1/2), and c-JUN NH₂-terminal kinase (JNK) [15]. These three proteins participate in regulating a large number of signaling pathways, including those involved in cell survival, apoptosis, adaptation, differentiation, and proliferation [16, 17]. MAPK family members are activated by different cell types, stress sources, and with different activation time [18]. The phosphor-MAPKs are closely involved in apoptosis induced by stress stimuli, such as release of cytokines, irradiation, heat stress, osmotic stress, ischemia, and reactive oxygen species (ROS) [19]. In addition, V is also able to activate the MAPK family members to induce apoptosis in many different cell types [20].

We hypothesized that V influences the phosphorylation of MAPKs to mediate

mitochondrial dysfunction, then the caspase family is recruited and activated, eventually leading to apoptosis. The purpose of this study was to utilize a previously established V^{5+} -induced apoptosis model to investigate whether the V-induce apoptosis in OMECs is related to the MAPK-mediated mitochondrial signaling pathway.

2. Materials and Methods

2.1 Ethics Statement

This study was undertaken in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the State Council of the People's Republic of China. The experimental protocol used in the study was approved by the Animal Care and Use Committee of Sichuan Agricultural University.

2.2 Isolation and Culture for OMECs

To establish a primary culture system for OMECs, the method for oviduct cell dissociation was slightly modified from that of Jung's and Kasperczyk's studies [21, 22]. Briefly, the oviducts were selected from 16-wk-old Lohman layers. The magnum segments of oviducts were trimmed free of blood and connective tissue, then the magnum was dissected into a 1 mm³ tissue sample by a sterile ophthalmic scissor, and washed in 37°C preheated PBS (Hyclone, Logan, UT, USA) until liquid

clarification. Consequently, the tissue samples were incubated for 70 min in a water bath at 37°C with 1 mg/kg collagenase IV (Sigma, St. Louis, MO, USA). Then, the dispersed cells were filtered through a 40-µm nylon cell strainer (BD Falcon, Franklin Lakes, NJ, USA). The filtrate was centrifuged at 700 rcf for 20 min and the collected cells were suspended into growth media and cultured in an incubator (3111, Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO₂ at 37 °C.

2.3 Experimental Design

Then cultured OMECs were allocated to 8 treatments as follows: 1) control, containing 0 µmol/L V (ammonium metavanadate, Sigma); 2) V100, containing 100 µmol/L V; 3) V100+SB203580 (P38 inhibitor, CST, Danvers, MA, USA); 4) SB203580; 5) V100+U0126 (ERK1/2 inhibitor, CST); 6) U0126; 7) V100+SP600125 (JNK inhibitor, CST) and 8) SP600125. Both V and the different MAPKs inhibitors were dissolved in DMEM-F12 (serum-free medium). OMECs were exposed for 12 h to V after pretreating them with 10 µM SB203580 and U0126 for 2 hours, or 50 µM SP600125 for 40 min, respectively.

2.4 Flow Cytometry for apoptosis

OMECs were seeded in 12-wells plates at a 5×10^4 density of cells, one well of cells was pooled together as a sample and six samples per treatment were collected. After 12 h exposure to V, the cells were digested by adding 0.125% trypsin (Hyclone)

and were incubated for 4 mins at 37°C; the OMECs cells were then centrifuged (1000 rpm for 10 min), and washed with cold PBS twice to remove residual trypsin. The collected cells were double stained with fluorescein isothiocyanate (FITC)-labeled annexin V (Invitrogen) and propidium iodide (PI; Invitrogen). Finally, OMECs apoptosis was detected by a flow cytometer (BD FACSVerse™ Flow Cytometer 651154, Germany) following the manufacturer's instructions.

2.5 Fluorescence Microscope and Microplate reader for $\Delta\psi_m$ Assay

Six samples for each treatment were assayed for $\Delta\psi_m$ by a Mitochondrial Membrane Potential assay kit (Beyotime, Beijing, China). Briefly, cells were incubated with a JC-1 stain solution for 60 min at 37°C with 5% carbon dioxide (CO₂). Then cells were washed twice with a JC-1 stain buffer (1×), and 500 µl JC-1 stain buffer (1×) was added into every well. Two images were simultaneously captured by means of a fluorescence microscope at the excitation wave length of 505 nm and 575 nm, respectively. Finally, these two captured images were merged by the NIS-Elements F 3.2 image software. Cells were then collected with cell scrapers from every well and were suspended in the JC-1 stain buffer (1×). JC-1 monomer was measured by a microplate reader (SpectraMax M2, Molecular Devices, USA) at 490 nm excitation wave length and 530 nm emission wave length, for the evaluation of JC-1 aggregates at 525 nm excitation wave length and 590 nm emission wave

length. Finally, the ratio of fluorescence values between JC-1 aggregates and JC-1 monomer was calculated.

2.6 Lactic dehydrogenase activity

OMECs were seeded in 6-well plates at a 1×10^5 density of cells, and the supernatant of one well was pooled together as a sample, and 6 samples were collected for each treatment. Lactic dehydrogenase (LDH) activity in the supernatant was measured by means of a commercially available kit following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Xuanwu, Nanjing, China). Then, the absorbance value of LDH activity was measured by a microplate reader at 450 nm wave length.

2.7 Total RNA Extraction and RT-PCR

Total RNA from OMECs was extracted using TRIzol reagent (Takara, Kusatsu, Shiga, Japan), and RNA contraction was determined by a spectrophotometer (NANADROP 2000, Thermo, USA). We used PrimeScript RT reagent kits (Takara) to reverse-transcribe 600 ng of RNA from each sample in a total volume of 20 μ l of single-stranded cDNA by using T100TM Thermal Cycler (BIO-RAD, USA), which contained 2 μ l gDNA Eraser Buffer, 1 μ l gDNA Eraser, 1 μ l PrimeScript RT Enzyme MixI, 4 μ l 5xPrimeScript Buffer, 1 μ l RT Primer Mix and the content of RNase free dH₂O was determined by the volume of RNA. According to manufacturer's

instructions, the 1 µl cDNA, 0.4 µl forward (10 µM) and 0.4 µl reverse primers (10 µM, as shown in Table 1), 3 µl deionized water were mixed with the SYBR[®] Premix Ex Taq II kits (Takara) and Ct values were measured by the Standard Real-Time PCR System (ABI 7900HT, Applied Biosystems, USA). A relative quantification of gene expressions were calculated after the nonspecific control β-actin transcript as a calibrator using $2^{-\Delta\Delta C_t}$ method.

2.8 Statistical Analysis

All data are available and presented as means ± SEM. All statistical analyses were performed using ANOVA (SAS 9.0). The means were compared using Turkey's multiple-comparisons test to determine the significance of the differences among treatments. $P < 0.05$ was considered statistically significant.

3. Results

3.1 MAPKs Inhibitors Alleviated V-induced Apoptosis in OMECs.

Using flow cytometry and calculating the sum of values in late apoptosis and early apoptosis, we found that the apoptosis rate was below 10% in the control group (Fig. 1A). The apoptosis rate increased to $36.11 \pm 1.8\%$ after treatment with 100 µmol/L V (Fig. 1B), indicating early apoptosis. Whereas when OMECs were pretreatment with MAPK inhibitors, V-induced apoptosis was less than 30% (Fig. 1C, 1E, and 1F). However, the apoptosis rate was about 10% when OMECs were not

exposed to V and treated with a single MAPK inhibitor only (Fig. 1D, 1F, and 1H). To investigate the effects of V and MAPKs inhibitors on apoptosis in OMECs, the data from repeated experiments were summarized and analyzed. As shown in Fig. 1I, the apoptosis rates of cells after V100 treatments were greater than after all the other treatments ($P<0.05$). Cells pretreated with any MAPK inhibitor, but without V, did not differ significantly from the control cells, suggesting that the MAPK inhibitors had no effect on apoptosis, except in cells treated with 100 $\mu\text{mol/L}$ V, which alleviated V-induced apoptosis in the OMECs ($P<0.05$).

3.2 Lactate dehydrogenase

Compared with the control, V100 exposure dramatically increased LDH activity in the supernatant ($P<0.05$), reaching the highest values (275.8 ± 33.6 U/gprot) among all treatments (Fig. 2). The LDH activity was significantly higher after V100 treatment than after treatment with any single MAPK inhibitor+V100 treatment ($P<0.05$). However, treatments with MAPK inhibitors alone, without V exposure, had little effect on LDH activity. These results show that each of the MAPK inhibitors suppressed the adverse V-induced effects on LDH activity in OMECs.

3.3 P38 and ERK1/2 reduced V-induced depolarization of $\Delta\psi\text{m}$ in OMECs.

Depolarized (green) and polarized (red) mitochondrial membranes were

detected using an inverted fluorescence microscope. The merged images showed that in the control and in the SB203580 and U0126 treatments color red were prevalent, suggesting a higher formation of JC-1 aggregates and a higher $\Delta\psi_m$. The images collected from the V100+SB203580 and V100+U0126 treatments were less red than the control, and single MAPK inhibitor treatment, without V (Fig.3a, 4c, 4d, 4e, and 4f). In addition, images from the V100 and SP600125 treatments showed a higher prevalence of green suggesting that a higher formation of JC-1 monomers occurred and that $\Delta\psi_m$ decreased (Fig.4b, 4g, and 4h). As shown in Fig. 4, the depolarization ratio in the control was the highest, but the ratio was significantly decreased in the V100 group ($P<0.05$). Pretreatments with SB203580 and U0126 prevented the V100-triggered decreases in the ratio of JC-1 aggregate/JC-1 monomer ($P<0.05$). However, SP600125 not only failed to suppress the V100-induced decline of the ratio, but instead it reduced it further ($P<0.05$).

3.4 Effects of MAPKs inhibitors and V on cell apoptosis-related gene expression in OMECs

Compared with the mRNA expression levels of the examined genes in the control, set as standard 1, V100 downregulated Bcl-2 ($P<0.05$) (Fig. 6A) and upregulated Bax at the transcriptional level ($P<0.05$) (Fig. 5B). Pretreatments with individual MAPK inhibitors alone resulted in downregulation of the Bcl-2 mRNA

expression ($P<0.05$) and the V100-associated upregulation of the Bax mRNA ($P<0.05$) (Fig. 5B). As for CytC, we observed that V100 upregulated the CytC mRNA ($P <0.05$), and the pretreatment with either SB203580 or U0126 downregulated CytC mRNA expression in OMECs when treated with 100 $\mu\text{mol/L}$ V ($P<0.05$). However, when OMECs that were pretreated with SP600125, there was no downregulation of CytC mRNA expression compared with the V100 treatment group (Fig. 5C). Gene expression of Bax, CytC, caspase 3, 8, 9, Apaf1 and PPAR γ were increased in the V100 group compared with the control ($P<0.05$), while pretreatment with SB203580, U0126, or SP600125 was able to attenuate the upregulation of Bax, caspase 3, 8, 9, Apaf1 and PPAR γ expression induced by V100 ($P<0.05$), while MAPK inhibitors had little effect on Bax, caspase 3, 8, 9, Apaf1 and PPAR γ expression at the transcriptional level (Fig.5B, D, E, F, G and H). V100 also decreased PARP1 mRNA expression ($P<0.05$), but this effect was partially suppressed to some extent by after pretreating OMECs with SB203580 ($P<0.05$) (Fig. 6I).

3.5 Effects of MAPKs inhibitors and V on phosphor-MAPK protein expression in OMECs

Treated cell with V had a significant increase in protein level of p-P38 MAPK, p-ERK1/2, and p-JNK compared with that of the control group (Fig. 6, $P<0.05$). In

contrast, pretreated cells with JNK inhibitor (SP600125) can prevent increases in p-P38MAPK, p-ERK1/2, and p-JNK protein level triggered by V ($P < 0.05$). Similarly, P38 MAPK inhibitor (SB203580) and ERK1/2 inhibitor (U0126) had a similar response to p-P38 and p-JNK.

4. Discussion

The regulation of the intrinsic pathway of apoptosis is generally mediated by mitochondria. Many physical and chemical stimuli can cause mitochondrial dysfunction, resulting in $\Delta\psi_m$ depolarization, which suggests that the mitochondrial membrane has become permeable or has ruptured; then CytC is released into the cytosol, binds to Apaf-1, caspase 9 is recruited, it activates caspase 3, leading to apoptosis [23]. This study shows that V100 was able to increase apoptosis in the OMECs cells, but pretreatment with a P38, ERK1/2 or JNK inhibitor reduced the V-induced apoptosis rate to some extent. Moreover, V induced apoptosis in the JB6 P+ mouse epidermal cell line by activating ERK and JNK, but not the P38 pathway [24]. These observations further indicate that MAPK family members may be involved in the V-induced apoptosis and that the different MAPK family members may play various roles in the apoptosis of different cell types. LDH activity is directly proportional to LDH release, which indicates the integrity of the cells. Excessive LDH release indicates that the permeability of the cell membrane is

increased or that it has even ruptured, implying that apoptosis or necrosis has occurred [25]. Similarly, to the observations made on OMECs apoptosis, the V-increased LDH activity in the supernatant was significantly reduced by pretreatment with MAPK inhibitors, which further demonstrates that the V-induced apoptosis in OMECs was mediated by MAPK.

Cells contain both pro- and anti-apoptotic proteins, which can be regulated by phosphorylation of the MAPK family. When cells are subjected to serious stress, the phosphor-MAPKs increase the expression levels of pro-apoptotic proteins and reduce the expression levels of anti-apoptotic proteins, triggering the apoptotic pathway [26-29]. The phosphor-MAPK can change the ratio of Bax to Bcl-2. In this study, V100 upregulated the pro-apoptotic protein Bax mRNA levels and downregulated the anti-apoptotic protein Bcl-2 mRNA levels. Our findings are supported by the recent observations made in HaCaT cells where V was able to increase the Bax/Bcl-2 ratio resulting in apoptosis [30]. In our previous study, we demonstrated that V was able to trigger the phosphorylation of P38, ERK1/2 and JNK (In press). It has been reported that when pro-apoptotic protein levels are enhanced, this can lead to the $\Delta\psi_m$ depolarization, increasing the permeability of the mitochondrial membrane and the opening of permeability transition pores (PTP); consequently, CytC can be released from the mitochondria into the cytosol [31, 32].

In this study, V increased the depolarization of $\Delta\psi_m$ in OMECs, suggesting that CytC in mitochondria was most likely released into the cytoplasm. Moreover, the increase in green coloration (JC-1 monomers) in the merged images indicates that the $\Delta\psi_m$ depolarization was severe, implying that early apoptosis was occurring, supporting our apoptosis results. A similar study reported that V induced the depolarization of $\Delta\psi_m$ in hepatic cells [33]. Although P38 and ERK1/2 inhibitors reduced V-induced depolarization of $\Delta\psi_m$, the JNK inhibitor did not have this effect. It could be argued that while P38 and ERK1/2 participate in the mitochondrial pathway that regulate apoptosis in OMECs exposed to V, JNK mediates are involved through another pathway. In addition, the results of this study indicated that only P38 and ERK1/2 inhibitors decreasing CytC mRNA expression after treatment with V, while this was not the case when OMECs were treated with the JNK inhibitor. Moreover, in the present study, V increased the phosphorylation of P38, ERK1/2, and JNK in OME cells, but P38, JNK, and ERK1/2 inhibitors suppressed the phosphorylation of these proteins. Other studies have also reported that V activates ERK1/2 and P38, and inhibitors of ERK1/2 or P38 significantly alleviated V-induced oxidative stress in human lung fibroblasts and airway epithelial cells [34,35]. Therefore, our findings bring further evidence to the V narrative, in that P38 and ERK1/2 phosphorylation are the result of the increase in the Bax/Bcl2 ratio, causing

$\Delta\psi_m$ depolarization, which then results in CytC to be released from mitochondria into the cytosol.

The data on genes downstream of the mitochondrial pathway, showed that V increased caspase 3 and 8 mRNA levels and that pretreatment with P38 and ERK1/2 inhibitors was able to partially reverse this effect, in agreement with previous studies [36, 37]. Caspase 3 mRNA expression increased in OMECs exposed to V100, probably as a consequence of CytC being released from mitochondria into the cytoplasm. Apaf-1 is a key regulator in the intrinsic (mitochondria-mediated) apoptotic pathway, which oligomerizes in response to cytochrome c release and forms a large complex known as apoptosome. Caspase 8 and 9, initiator caspase in the mitochondrial pathway, are recruited and activated by the apoptosome leading to downstream caspase-3 processing [38]. CytC then bound Apaf-1 in the cytoplasm which recruited caspase 9 and subsequently caspase 3 was activated. Thus, the caspase 3, caspase 8, 9 and Aapf1 mRNA expression level increased significantly in current study. As a target the gene of caspase 3, PARP1 mRNA level was upregulated after V100 exposure. PARP1 is a DNA repair enzyme, and the upregulation of caspase 3 expression destroyed the structure of PARP1 in cells. When cleaved, PARP1 mRNA expression decreased in OMECs, therefore the damaged DNA could not be efficiently repaired, eventually resulting in apoptosis.

The JNK inhibitor also reduced V-induced apoptosis. Although the JNK inhibitor had little effect on mitochondria, it upregulated caspase 3 mRNA expression in OMECs treated with V100, indicating not only that V-induced apoptosis is involved in the mitochondria mediated intrinsic pathway, but it is involved with the cell membrane receptor (Fas) of the extrinsic pathway. It has been reported that the phosphorylation of JNK activated activator protein 1 (AP-1). Activator protein 1 (AP-1) is a transcription factor that regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, and bacterial and viral infections. AP-1 controls a number of cellular processes including differentiation, proliferation, and apoptosis. As a promoter of FasL, AP-1 expression increases and then binds to the transmembrane protein Fas, which recruits caspase 8 to activate caspase 3, resulting in apoptosis [39].

Another result in this study showed that V can upregulate PPAR γ mRNA levels. Wang [40] reported that the activated PPAR γ was related to oxidized low density lipoprotein-induced apoptosis in vascular smooth muscle cells. Yang [41] also reported that PPAR γ gene silencing suppressed cisplatin-induced A546 cell apoptosis through the upregulation of Bcl-2 mRNA expression, which demonstrated that the activated PPAR γ promoted cell apoptosis. Moreover, there are interactions between PPAR γ and AP-1 [42, 43], which suggests that PPAR γ is involved in the extrinsic

pathway that regulates V induced apoptosis in OMECs.

5. Conclusion

These results indicated that MAPK can mediate V-induced OMEC apoptosis, which is regulated by three members involved in the different pathways. V exacerbate ROS generation in OMECs to activating MAPK family; the activation of p-P38 and p-ERK1/2 may lead to a higher Bax/Bcl-2 ratio, resulting in $\Delta\psi_m$ depolarization. Consequently, permeability of the mitochondrial membrane is increase and it is accompanied by upregulation of CytC mRNA expression, suggesting that CytC is released from the mitochondria to the cytosol. CytC binds to Apaf-1 to recruit caspase 9, resulting in the activation of caspase 3. Then, PARP1 is cleaved by the activated caspase 3, and consequently PARP1 expression decreases. The cleaved PARP1 is unable to repair damaged DNA, leading to apoptosis. Additionally, phosphor-JNK may upregulate PPAR γ expression mediating OMECs apoptosis through the extrinsic pathway where Fas/FasL are involved (Fig. 7).

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were responsible for acquisition and interpretation of data, J.P.W is involved in drafting the manuscript and revising it critically for important intellectual content; while professor K.Y.Z given final approval of the version published. Dr P. C, Y. L, and X.B.M helped to revise the manuscript. X.M.D, S.P.B, Q.F.Z, and H.W.P agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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