ULTRA STRUCTURE CHANGES OF DIOSGENIN-TREATED HUMAN MONOCYTE U937-DERIVED MACROPHAGES INDUCED BY *NAEGLERIA FOWLERI* LYSATE

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Abstract. Diosgenin has anti-amoebic activity against *Naegleria fowleri* and anti-inflammatory activity in human monocyte U937-derived macrophages incubated with *Naegleria* lysate. In order to better understand the mode of action of diosgenin, human monocyte U937-derived macrophages were induced by *N. fowleri* lysate and investigated using scanning electron microscopy (SEM). SEM revealed morphological changes of human monocyte U937-derived macrophages in size, shape, cytoplasmic extensions and microvilli. The formation of the disrupted membrane structure occurred in a time-dependent manner. No significant differences in cell morphology were observed between diosgenin-treated samples and untreated cells; however, macrophages induced by *N. fowleri* lysate or lipopolysaccharide (LPS) were completely damaged by 48 hours of incubation. Diosgenin-treated macrophages induced by *N. fowleri* lysate and LPS had thorn-like protrusions from filopodia and lamilipodia. Our findings show the affect of diosgenin at the ultracellular level.

Keywords: diosgenin, *Naegleria fowleri* lysate, human monocyte U937-derived macrophage, scanning electron micrograph

INTRODUCTION

The pathogenic amoeba *Naegleria fowleri* has a worldwide distribution and can cause acute primary amoebic meningoencephalitis (PAM) and severe central nervous system (CNS) diseases in animals and humans and is sometimes considered a neglected tropical disease (NTD) (Marciano-Cabral *et al*, 2009; Grace *et al*, 2015). *N. fowleri* infections have been documented in children and young adults after accidental exposure to contaminated recreational, domestic or environmental water sources (Marciano-Cabral, 2009, Dunn *et al*, 2016). *N. fowleri* has been thought to infect the human body by entering the host through the nose when water is splashed or forced into the nasal cavity (Siddiqui *et al*, 2016). Infection begins with attachment to the nasal mucosa, followed by locomotion along the olfactory nerve and through the cribriform

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plate to the olfactory bulbs in the central nervous system (CNS) (Grace *et al*, 2015).

The amoeba destroys neurons through contact-dependent and non-contact dependent mechanisms (Tiewchareon *et al* 2008a,b; Kim *et al*, 2008; Rabablert *et al*, 2016). Pathogenicity occurs via the formation of pseudopodia and food-cup structures (Cho *et al*, 2003). The destruction of tissue and hemorrhage necrosis of the brain is accompanied by inflammatory infiltration with neutrophils, eosinophils and macrophages (Marciano-Cabral, 2007; Cervantes-Sandoval *et al*, 2009). The infected microglial cells secrete pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α) in the brain (Kim *et al*, 2016). The release of pro-inflammatory cytokines from stimulated-leukocytes causes acute damage to neurons and necrotizing meningoencephalitis and can lead to death (Grace *et al*, 2015).

Macrophages have been shown to be important components of the host defenses (Weiss and Schaible, 2015). Lipopolysaccharide (LPS) is an outer membrane component of gram-negative bacteria, and is a potent activator of monocytes and macrophages (Williams and Ridley, 2000). Previous reports have demonstrated that LPS induced apoptosis in bone marrow derived macrophages (Xaus *et al*, 2000), human leukemia U937 cells (Suzuki *et al*, 2004), human monocyte U937-derived macrophages (Rabablert *et al*, 2016).

Diosgenin, a steroid sapogenin, obtained from *Dioscorea alata*, *Smilax china*, *Trigonella foenum graecum* and *Momordica charantia* plants, is used in traditional medicine to treat a variety of medical conditions (Danial *et al*, 2014, Jesus *et al*, 2016). Diosgenin has been reported to have antimicrobial activity (Pabon *et al*, 2013), anti-fungal activity (Nelson, 2009) and anti-amoebic activity (Rabablert *et al*, 2015). Diosgenin has been reported to have less toxicity against mammalian cells at therapeutic levels than amphotericin B, the drug currently used to treat *N. fowleri* infections (Rabablert *et al*, 2015; Jesus *et al*, 2016).

A previous study reported diosgenin has anti-inflammatory activity in human monocyte U937-derived macrophages after the macrophages have been stimulated with LPS or *N. fowleri* lysate by blocking TNF-alpha (α) synthesis (Rabablert *et al*, 2016). However, the effects of diosgenin on macrophage morphological changes induced by *Naegleria* lysate have not been reported in detail. In the present study, we investigated the morphological changes of diosgenin-treated macrophages, following stimulation with *Naegleria* lysate, as seen under scanning electron microscopy (SEM).

MATERIALS AND METHODS

Reagents

The diosgenin used in our study was donated by Miss Natchagorn Lumlerdkij, Center for Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. A stock solution of diosgenin was prepared using 1 gram of diosgenin dissolved in 5 ml dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO), to obtain a final diosgenin concentration of 200 mg/ml; the solution was then stored at -20°C until use. Lipopolysaccharide (LPS) from *Escherichia* coli strain 0127:B8 was purchased from Sigma-Aldrich. A stock solution of LPS was prepared using 1 mg of LPS dissolved in 8 ml distilled water (Thai Otsuka, Bangkok, Thailand) to give

a final LPS concentration of 125 μg/ml; this was used as a stock solution for LPS; it was also stored at -20°C until use.

***Naegleria fowleri* lysate**

N. fowleri trophozoites (Siriraj strain) were axenically cultured at 37°C in Nelson's medium supplemented with 5% heat-inactivated fetal calf serum (FCS) (Invitrogen, Carlsbad, CA) (Tiewchareon *et al*, 2014). Trophozoites were harvested after incubation at 4°C for 10 minutes by scraping, and then centrifuging at 500*g* (Thermo Scientific, Lougborough, England) for 30 minutes. The obtained pellet was resuspended in pre-chilled Roswell Park Memorial Institute (RPMI) 1640 Medium at a ratio of 1:4 and incubated on ice for 10 minutes. The cell suspension was sonicated with 10 short bursts of 10 seconds each, followed by an interval of 3 minutes for cooling. Cell debris was removed by centrifugation at 4°C for 30 minutes at 500*g*. The *N. fowleri* trophozoite protein concentration was measured by a Nanodrop 1000 spectrophotometer (Thermo Scientific, Lougborough, England). *Naegleria* lysate (40 mg/ml) was stored at -80°C until use.

Cell culture

For our study, we used a human monocyte U937 cell line donated by Professor Prasert Auewarakul, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The cells (2×10^5 cells) were cultured on a 35-mm petri dish (Corning, New York, NY) containing RPMI-1640 medium (Gibco-BRL, Grand Island, NY) containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and supplemented with 10% FCS, 2 mM glutamine, and 110 mg/ml sodium pyruvate, as described previously with some modifications (Ghosh *et al*, 2010). The U937 cells were differentiated into macrophage-like cells by treating with 250 ng/ml phorbol-12 myristate acetate (PMA, Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced after 4-days-culture.

Scanning electron microscopy

For a positive control group, *N. fowleri* lysate and LPS were used. For a negative control group, human monocyte U937-derived macrophages (1×10^6 cells) were cultured in a 35-mm petri dish containing RPMI-1640 supplemented with 10% FCS in the presence of diosgenin at a maximum non-toxic dose of 100 μg/ml (Rabablert *et al*, 2016) or RPMI-1640 medium alone for 1 hour, then LPS was added to the monocyte cells treated with diosgenin.

The experimental group was pre-incubated with diosgenin or medium alone, the cells were treated with *N. fowleri* lysate at 50% of the cytotoxic concentration (CC_{50}, 8 mg/ml) (Rabablert *et al*, 2016) or LPS (5 μg/ml) and incubated at 37°C in a 5% CO_2 incubator. Macrophages were harvested at 12, 24 and 48 hours of incubation and processed for scanning electron microscopy (SEM). Specimen preparation for SEM was performed as previously described (Brunk *et al*, 1981). Briefly, the selected cells were pre-fixed in 2.5% glutaraldehyde and 0.1 M phosphate-buffered saline (PBS) at a pH of 7.4, pre-warmed to 37°C, and kept at 37°C for 30 minutes before being cooled to 4°C and kept at that temperature for 24 hours. The untreated and treated cells were then fixed in 1% osmium tetroxide (OsO_4) and 0.1 M PBS at a pH of 7.4 at room temperature (RT) for 90 minutes, before being rinsed with 0.1 M PBS, sequentially dehydrated in serial dilutions of ethanol (25%, 50%, 75%, 95% and 100%), critical point dried, and then coated with gold-palladium

(Au-Pd). Finally, the untreated and treated cells were examined and photographed under a scanning electron microscope (SEM) (Hitachi S-51, Tokyo, Japan) at an accelerating voltage of 25 kilovolts (kV).

Statistical analysis

Statistically significant differences between groups were determined using the Student's *t*-test. A p-value < 0.05 was considered statistically significant.

RESULTS

We studied the effect of diosgenin on human U937-derived macrophages induced by *Naegleria* lysate, on the morphological characteristics of the macrophages at 12, 24, and 48 hours exposure. The studied human U937-derived macrophages had marked variations in size, shape, and spreading. Small ruffles (arrow, Fig 1a,b) were occasionally observed on the surfaces of the macrophages at 12 and 24 hours. Radial filopodia with thread-like characteristics (arrow, Fig 1c) and more extensive ruffling of the peripheral membrane were observed at 48 hours of incubation. Diosgenin-treated macrophages also had marked variations in size, shape, spreading, and filopodia (arrow, Fig 1d) similar to untreated macrophages at 12 hours. Diosgenin-treated macrophages spread out using their frilly cytoplasm as arms to draw themselves along through the substrate and to seek and engulf particles. Diosgenin-treated macrophages were slightly elongated in shape, had numerous cytoplasmic extensions and phagocytic cups or phagosomes (arrow, Fig 1e,f) at 24 and 48 hours of incubation.

Naegleria lysate-induced macrophages were rounded, had crater formation and early cracking on the surface of the peripheral membrane. Remnants of engulfed materials were observed in the craters (arrow, Fig 2a). Macrophages lacked microvilli and were irregularly shaped, and had multiple membrane wrinkles at 12 hours of incubation (Fig 2a). Activated macrophages were condensed, and had cracked surface membranes (arrow, Fig 2b), at 24 hours of incubation. Necrotic macrophages had swollen and ruptured membranes resulting in destruction (Fig 2c) at 48 hours. The LPS-induced macrophages (5μg/ml) had knot-like structures (arrow, Fig 2d) in the cellular periphery at 12 hours of incubation. LPS-induced macrophages had rounded surface protuberances characteristic of budding (arrow, Fig 2e), a well-known feature of apoptosis at 24 hours of incubation. Macrophages stimulated by LPS underwent cell death (arrow, Fig 2f) by 48 hours of incubation.

Diosgenin-treated macrophages stimulated with *N. fowleri* lysate were round and had small protruding thorn-like processes (arrow, Fig 3a) at 12 hours of incubation. Cells with large protruding thorn-like processes (arrow, Fig 3b,c), and distorted membranes were seen at 24-48 hours of incubation. Diosgenin-treated macrophages stimulated with LPS were round, similar in size and had filopodia (arrow, Fig 3d,e) similar to diosgenin-treated macrophages stimulated with *N. fowleri* lysate at 12 and 24 hours of incubation. LPS-stimulated-macrophages treated with diosgenin were small, but had large lamellipodia (arrow, Fig 3f), filopodia and ruffling membranes.

DISCUSSION

Diosgenin has anti-amoebic activity against *Naegleria fowleri* trophozoites (Rabablert *et al*, 2015) and has anti-inflammatory effects on macrophages (Rabablert *et al*, 2016). In this study, we investigated the effects of diosgenin on human monocyte

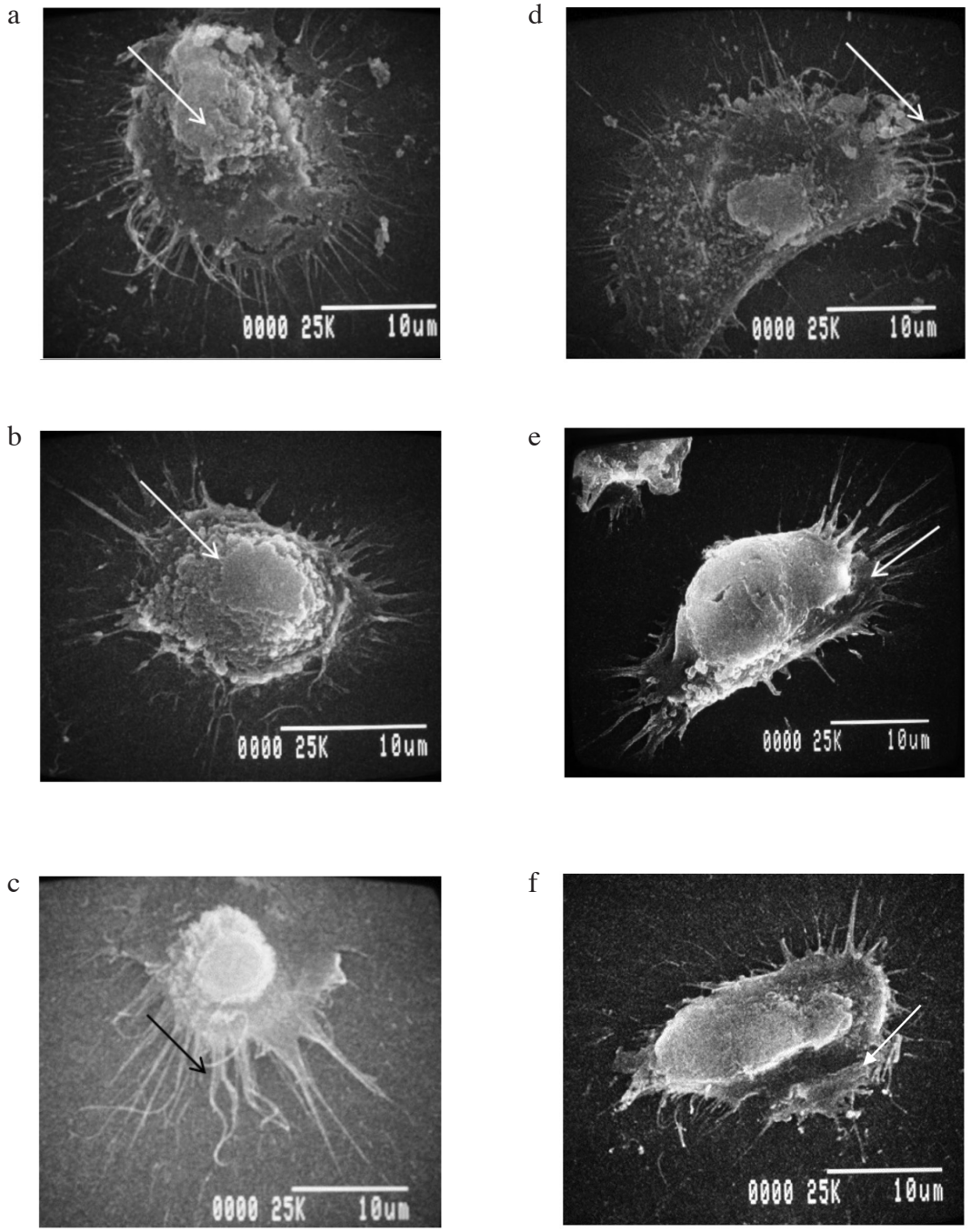

Fig 1–A scanning electron microscopy of human U937-derived macrophages after treatment with RPMI-1640 medium (a,b,c) and diosgenin (100 mg/ml) (d,e,f) for 12, 24, 48 hours of incubation, respectively. Arrows indicate membrane ruffles (a,b); radial filopodia (c); filopodia (d); a phagostome and an elongated cell (e,f). The bar represents 10 μm.

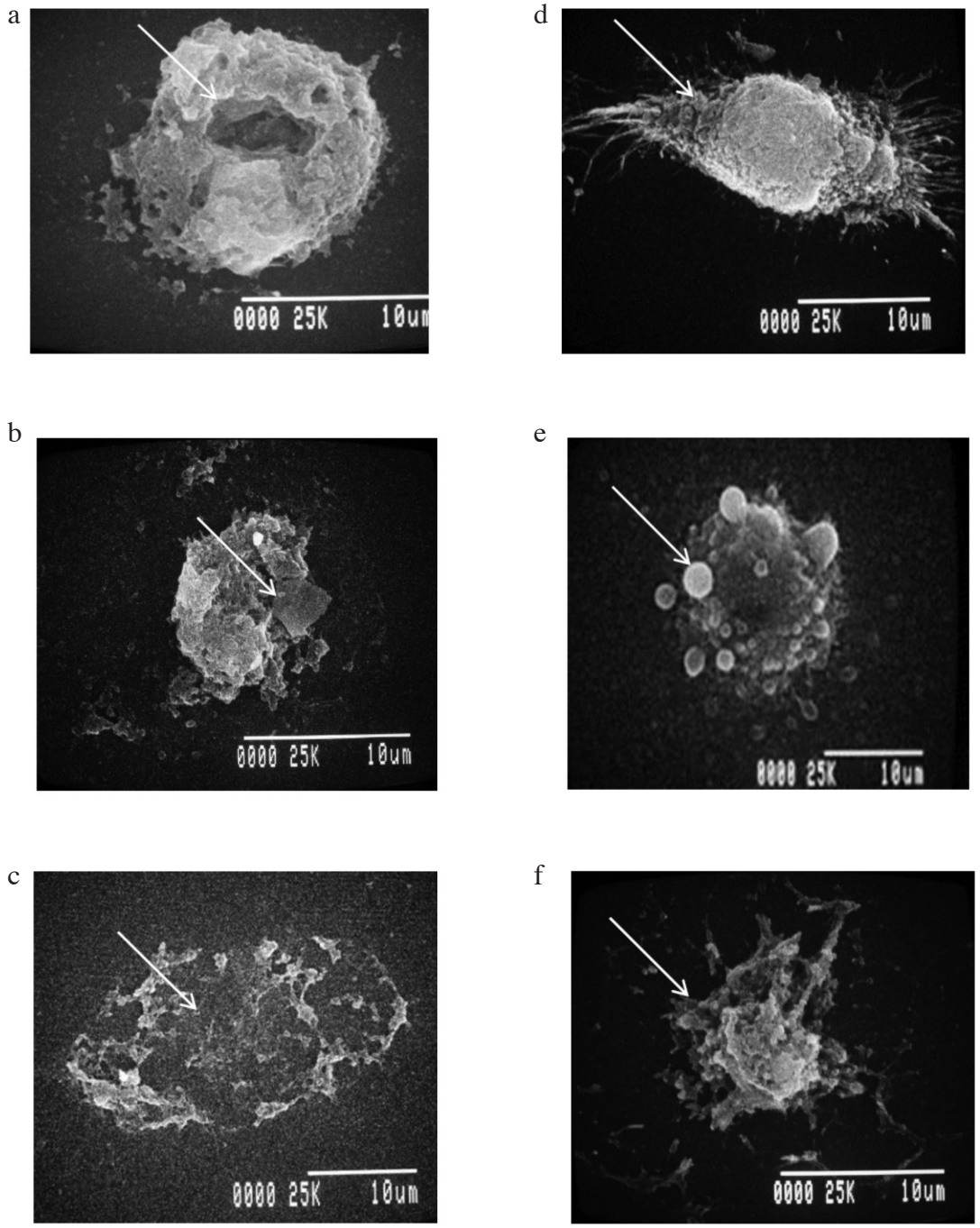

Fig 2–Scanning electron microscopy of human U937-derived macrophages after stimulation with *Naegleria* lysate (8 mg/ml) (a,b,c) and lipopolysaccharides (5 mg/ml) (d,e,f) for 12, 24, 48 hours of incubation, respectively. Arrows indicate a crater (a); cracked surface(b); progressive destruction (c); knot-like condensation (d); protuberances characteristic of budding (e) and a necrotic cell (f). The bar represents 10 μm.

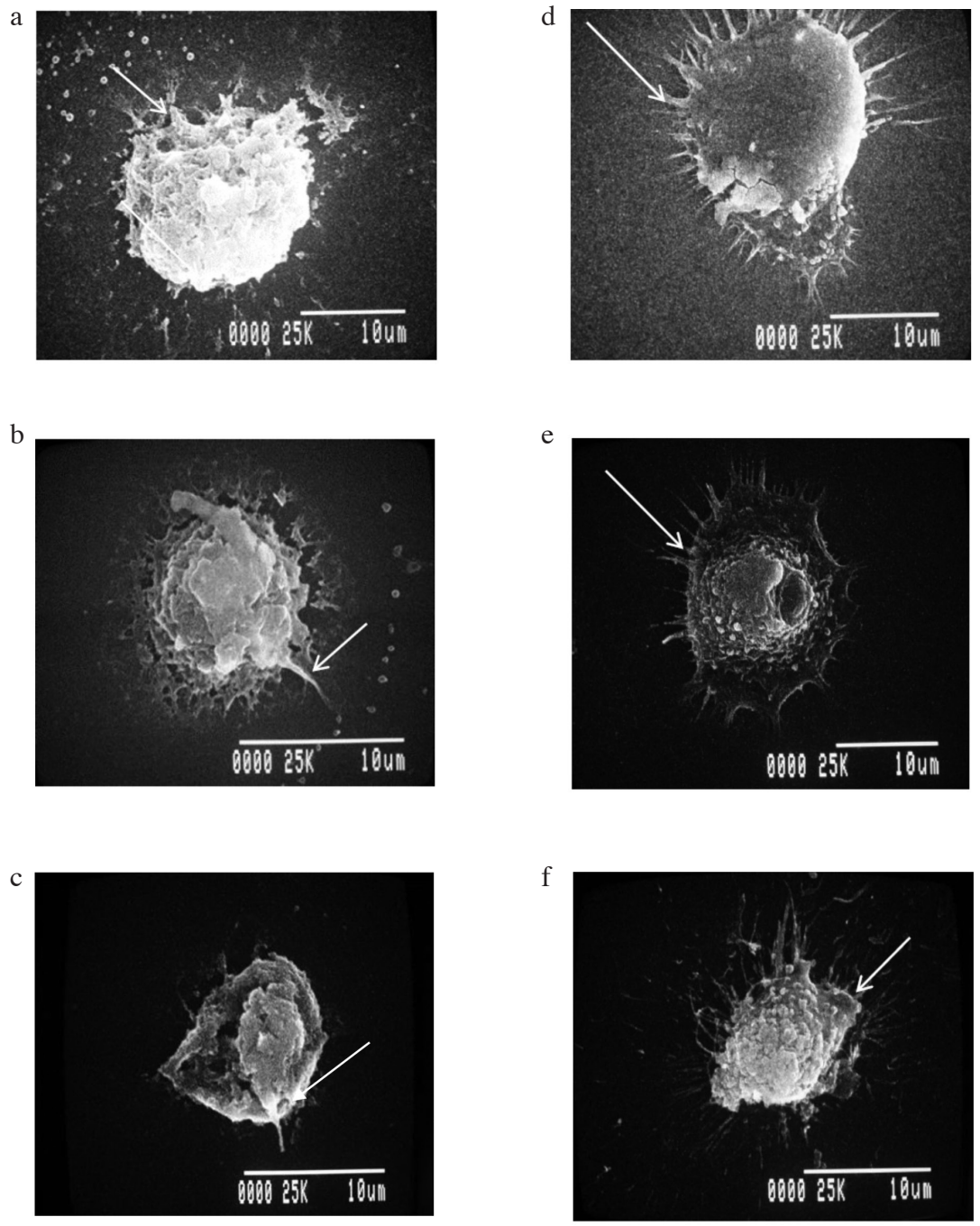

Fig 3–Scanning electron microscopy of diosgenin-treated human U937-derived macrophages after stimulation with *Naegleria* lysate (8 mg/ml) (a,b,c) and lipopolysaccharides (5 mg/ml) (d,e,f) at 12, 24, 48 hours of incubation, respectively. Arrows indicate an early torn-like process (a); complete torn-like process (b,c); filopodia (d,e), lamillipodia (f). The bar represents 10 μm.

U937-derived macrophages after stimulation with *Naegleria* lysate compared with LPS at 12, 24, and 48 hours of incubation by SEM.

Macrophages at 12 hours incubation were spherical and had microvilli, numerous filopodia and some membrane ruffling (Fig 1a,b,c). Our findings are similar to a study by Williams and Ridley (2000) who found the size, shape and spreading of human Bac1 macrophages changed in a time-related manner. The radial filopodia of macrophages are used for locomotion within the tissues to enable the cells to travel and engulf foreign particles (Mattilia and Lappalaiman 2008; Venter *et al*, 2014). Diosgenin-treated macrophages in our study had marked variations in size, shape, and degree of spreading, similar to the control of macrophages at 12, 24, and 48 hours of incubation (Fig 1d,e,f). Diosgenin has been found to induces cytotoxicity and autophagy in K562 and BaF3-WT cells; the autophagy was cytoprotective (Jesus *et al*, 2016). Diosgenin elevates reactive oxygen species (ROS) levels, and this oxidative stress produces cytotoxic effects on chronic myelogenous leukemia (CML) cells and induces autophagy (Jiang *et al*, 2016).

N. fowleri lysate caused progressive degeneration and death of macrophages in our study (Fig 2a,b,c). The cytotoxicity of *Naegleria* lysate on macrophages occurred in a time-dependent manner in our study and we observed necrotic macrophages. This finding is similar to a previous study which found *Naegleria* lysate induced apoptosis and necrosis of mouse microglia cells (Lee *et al*, 2011). LPS-induced monocytes and macrophages have been found to rapidly stimulate actin reorganization and promote adhesion and spreading (Doherty *et al*, 1989). In our study, LPS-induced macrophages developed knot-like structures (arrow, Fig 2d) at the cellular periphery by 12 hours incubation.

LPS-induced macrophages formed rounded surface protuberances characteristic of budding phenomena (arrow, Fig 2e), a well-known feature of apoptosis at 24 hours. This finding is different than that of Xaus *et al*, (2000) who found LPS induced apoptosis among bone marrow macrophages by 3 hours incubation with LPS treatment. In our study, macrophages stimulated with LPS developed a necrosis-like form of death (arrow, Fig 2f) by 48 hours.

These findings are similar to the treatment of LPS low dose (less than 5 μg/ml) resulted in cytostatic and cytotoxic effect in long-term culture (168 hours) THP-1 monocytes differentiation to macrophages and caused of cell death at higher doses (more than 10 μg/ml) (Mytych *et al*, 2017). Apoptotic cells have been found to be different from other cell types and cell lines (Suzuki *et al*, 2004). We propose LPS acts in a time-dependent manner and initiates apoptotic and necrotic effects.

Diosgenin-treated macrophages induced by *Naegleria* lysate developed protruded torn-like processes by 12 hours incubation (Fig 3a,b,c) and then developed completely torn-like processes by 24-48 hours incubation. Our findings are similar to a study by Kim *et al* (2016) who found necrosis and apoptosis of macrophages induced by *N. fowleri* trophozoites. Previous studies reported diosgenin induced anti-inflammatory activity against LPS-induced inflammation in mouse primary peritoneal macrophages (Jung *et al*, 2010) and mouse lung injuries (Gao *et al*, 20 13), similar to our finding in diosgenin-treated macrophage induced by LPS (Fig 3d-f). These findings suggest diosgenin may have anti-amoebic activity against *N. fowl-*

eri and anti-inflammatory activity against the cytotoxic effects caused by LPS.

In conclusion, our findings suggest diosgenin has an inhibitory effect on cytotoxicity and autophagy among human monocyte U937-derived macrophages induced by *Naegleria* lysate and LPS. Diosgenin may play a cytoprotective role in the survival of human U937-derived macrophages exposed to *N. fowleri* .

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