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Different roles of intracellular and extracellular reactive oxygen species of neutrophils in type 2 diabetic mice with invasive aspergillosis

Xianghua Xu, Chu Xia, Yi Huang*

Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital (Changhai Hospital), Naval Medical University, Changhai Road 168, Yangpu, Shanghai 200433, China

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ABSTRACT

Diabetic patients have an increased risk of invasive aspergillosis (IA), but the mechanism is still unclear. Reactive oxygen species (ROS) produced by neutrophils play a key role in defense against *Aspergillus* infection. Since diabetes mellitus affects the production of ROS from neutrophils, the purpose of this study is to investigate whether this effect is related to the susceptibility of diabetic mice to IA. C57BL/6 mice were used to establish type 2 diabetes mellitus (T2DM) model, and IA was induced by airway infection with *Aspergillus fumigatus*. After infection, the fungal load, neutrophil count and ROS content in the lung tissues of T2DM mice were higher than those in the control mice, and the inflammation of the lung tissue was more serious. After being exposed to hyphae in vitro, compared with the control group, neutrophils in T2DM mice had higher apoptosis rate and intracellular ROS content, as well as lower viability, extracellular ROS content and fungicidal ability. In summary, after T2DM mice are infected with *A. fumigatus*, the reduction of extracellular ROS produced by neutrophils may lead to a decrease in fungicidal ability, while the increase of intracellular ROS is related to neutrophil and lung tissue damage.

1. Introduction

Diabetes is a common disease. In 2019, the International Diabetes Federation (IDF) estimated that 463 million people in the 20–79 age group worldwide have diabetes, with a prevalence rate of 9.3 %, mainly T2DM (International Diabetes Federation, 2019). Diabetes leads to an increased risk of infection, which involves almost all pathogens (Dunachie and Chamnan, 2019; Carey et al., 2018). IA is a serious infectious disease caused by *Aspergillus*. There are more than 300,000 new cases of IA every year in the world, and the mortality rate is as high as 30–80 % (Bongomin et al., 2017). Large-scale studies have found that the prevalence of diabetes in patients with IA is 17.5–30.9 % (Rotjanapan et al., 2018; Sun et al., 2017; Taccone et al., 2015; Nivoix et al., 2008; Chakrabarti et al., 2019), which is much higher than that in the general population, suggesting that diabetes is a risk factor for IA.

At present, the mechanism of increased risk of IA in diabetic patients is not clear. Since many studies have found that IA is prone to occur in patients with neutropenia, the neutrophil count has long been regarded as a major and key indicator of susceptibility to IA (Patterson et al., 2016). However, IA also occurs in a large number of patients without neutropenia, which is particularly evident in patients with chronic granulomatous disease. The neutrophils of patients with chronic granulomatous disease are unable to produce effective ROS due to the abnormal function of NADPH oxidase 2 (NOX2), so these patients have a tendency to recurrent bacterial and fungal infections (Stasia and Li, 2008), which suggests that what really matters is not only the neutrophil count but also the ROS content produced by neutrophils. Many studies have shown that the ROS production of neutrophils is abnormal in diabetic patients (Hand et al., 2007; Wang et al., 2018). Considering the key role of ROS in the defense against *Aspergillus* infection, we speculated that abnormal ROS production of neutrophils may be an important cause of IA in diabetic patients.

In this study, the T2DM mouse model was established by high-fat diet and streptozotocin (STZ) injection, and the IA model was established by inoculating *A. fumigatus* via the airway. The neutrophil count and function changes of T2DM mice and the control mice after infection were compared, and the changes of ROS produced by neutrophils were focused on. We found that the reduction of extracellular ROS may lead to a decrease in the fungicidal ability of neutrophils in T2DM mice, while the increase of intracellular ROS may aggravates neutrophil and tissue damage.

* Corresponding author.
E-mail addresses: ccrysy@sina.com (X. Xu), xia.tianyu@163.com (C. Xia), huangliur@163.com (Y. Huang).

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2. Materials and methods

2.1. Materials

A. fumigatus strain (ATCC13703) expressing green fluorescent protein (GFP) was generously presented by Dr. Nancy Madinger (University of Colorado Health Sciences Center). Sabouraud medium, Tween-80 and STZ were purchased from Sigma-Aldrich (USA). High-fat (60 % of energy) feed (D12492) and low-fat (10 % of energy) feed (D12450 J) were purchased from Research Diets (USA). RPMI1640 medium and fetal calf serum (FCS) were purchased from Gibco (USA). Mouse TNF-α and IL-1β immunoassay kits were purchased from R&D (USA). MTT and XTT were purchased from Sigma-Aldrich (USA). Reactive oxygen species assay kit by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) chemofluorescence was purchased from Sigma-Aldrich (USA). Reactive oxygen species assay kit by lucigenin chemiluminescence was purchased from Sigma-Aldrich (USA). Anti-NOX2/gp91phox antibody (primary antibody) (ab180642), Anti-myeloperoxidase (MPO) antibody (primary antibody) (ab9535) and goat anti-rabbit IgG H&L (HRP) (secondary antibody) (ab6721) were purchased from Abcam (USA) and used for immunohistochemistry (IHC). Neutrophil separation solution kit was purchased from Miltenyi Biotec (Germany). Annexin V-FITC apoptosis Detection kit was purchased from BD (USA). Diphenylenene-iodonium chloride (DPI) was purchased from Sigma-Aldrich (USA).

2.2. Methods

2.2.1. Animal and T2DM model

5-week-old, male, C57BL/6 mice (weight 18 ± 2 g) were purchased from Shrek Experimental Animals Co., Ltd. (Shanghai, China). Five animals per cage were kept and animals were raised according to the standard of Guide for the Care and Use of Laboratory Animals (8th edition) (Council, 2011). The animals were adapted for 7 days before exposure. All animal experiments were approved by the Animal Research Committee of Changhai Hospital.

T2DM mouse model was established according to the method of Han et al. (2019). In short, mice were fed with high-fat feed for 4 weeks, and then intraperitoneally injected with 40 mg/kg STZ daily for 5 consecutive days. One week after STZ injection, mice with non-fasting blood glucose ≥250 mg/dl were diagnosed as diabetes (Leiter, 2009). The control mice were fed with low-fat feed and injected with the same dose of sodium citrate buffer instead of STZ. Mice were infected with *A. fumigatus* 2 weeks after STZ injection.

2.2.2. Infection with A. fumigatus

The conidia suspension was prepared according to the method of Dubourdeau et al. (2006). Briefly, *A. fumigatus* was inoculated on a Sabouraud solid medium. When the medium was full of colonies, the hyphae were removed by washing with PBS containing 0.1 % Tween-80, the conidia were suspended in PBS, and the concentration of the conidia suspension was adjusted to be 5×10^7 CFU (colony forming unit)/mL for standby. The preparation of *A. fumigatus* hyphae suspension referred to the method of Rohm et al. (2014). *A. fumigatus* conidia (1×10^6 CFU/mL) were incubated in Sabouraud liquid medium for 24 h. The hyphae were collected and washed by PBS. The hyphae were broken into 10 − 100 μm fragments by ultrasonic crushing method. The concentration of the prepared hyphae fragments was 1×10^6 CFU/mL.

A. fumigatus conidia were inoculated with a nasal drop method according to the method of Dubourdeau et al. (2006). Briefly, the mouse was anesthetized with ether and kept upright and slowly dripped into the nostrils 20 μl *A. fumigatus* conidia suspension at the concentration of 5×10^7/mL. After 2 h, each mouse was injected with hyphae fragments suspension containing 10,000 CFU by oropharyngeal irrigation with reference to the method of Rohm et al. (2014).

2.2.3. Fungal load

Fungal load was measured referring to the method of Teschner et al. (2019). Under aseptic conditions, 20 mg tissues in the upper, middle and lower fixed position of the right lung of the mouse were taken and crushed with 200 μl saline, and then suspensions of 10 times dilution series (1×10^1, 1×10^2 and 1×10^3 dilution) were prepared. 100 μl suspension was inoculated on Sabouraud medium with 3 plates per concentration, and incubated at 26.5 ℃ for 7 days in a mold incubator. According to the number of colonies on the medium, the colony number of *A. fumigatus* in the suspension was calculated: colony number/mL = the sum of (the average number of colonies in three dishes at each dilution concentration × dilution multiple) divided by 3.

2.2.4. Bronchoalveolar lavage

Bronchoalveolar lavage was performed according to the method of Bozza et al. (2009). Briefly, the trachea was exposed after the mouse was euthanized, and a 22 G vein intubation needle was inserted into the trachea and the trachea was washed with 0.8 ml PBS for 3 times. The bronchoalveolar lavage fluid (BALF) was centrifuged at 4 ℃ at 1500 rpm for 5 min, and the cells were resuspended with 1 ml PBS for neutrophil count. The neutrophil count was measured by an automatic microbiochemical analyzer (Becman, USA).

2.2.5. Neutrophil isolation and incubation

Circulating blood was collected after cardiac puncture. Neutrophils were purified by mouse peripheral blood neutrophil separation kit according to the manufacturer's instructions. The cells were resuspended in RPMI1640 medium and incubated in 5% CO_2 incubator at 37 ℃. The neutrophil count was measured by an automatic microbiochemical analyzer (Becman, USA).

2.2.6. Measurement of ROS

ROS in tissues and cells were detected by DCFH-DA chemofluorescence assay (Yang et al., 2018) according to the manufacturer's instructions (Sigma-Aldrich, USA). DCFH-DA is cell permeable and hydrolyzed to 2′,7′-dichlorodihydrofluorescein (DCFH) after uptake. DCFH is trapped inside cells and then oxidized to the fluorescent molecule 2′,7′-dichlorofluorescein (DCF). The fluorescence signals were read at 485 nm excitation and 525 nm emission by enzyme labeled instrument (Thermo, China). Since a variety of ROS can cause the conversion of DCFH to DCF, DCFH-DA is regarded as a non-specific probe (Tarpey and Fridovich, 2001). ROS in cell supernatants were detected by lucigenin Chemiluminescence assay, according to the manufacturer's instructions (Sigma-Aldrich, USA). Lucigenin is cell impermeable and thus can be used to detect extracellular ROS. Lucigenin is selective for superoxide ($O_2^{\cdot -}$) and reduced by $O_2^{\cdot -}$ to a lucigenin cation radical. The reaction mixture was put into the luminometer (Siemens, Germany) for reading at 37℃.

2.2.7. Cytokine detection

Mouse ELISA kits were used to measure TNF-α and IL-1β in cell supernatants and tissue lysates according to the manufacturer's instructions (R&D, USA).

2.2.8. Histology

According to the method of Rohm et al. (2014), the lung was dissected and fixed, and the fixed lung tissue was embedded in paraffin. Tissue sections were stained with hematoxylin and eosin. Fungi were observed under fluorescence microscope (Leica, Germany).

2.2.9. Detection of NOX2 and MPO in lung tissues

According to the methods of Ma et al. (2019) and Hsu et al. (2015), NOX2 and MPO in lung tissue specimens were detected by IHC. Paraffin-embedded lung sections were dewaxed and rehydrated prior to quenching of endogenous peroxidase and antigen retrieval, and then blocked. Primary antibody diluted 1:100 was used to incubate sections

overnight. Afterward, the sections were incubated with secondary antibody followed by diaminobenzidine and hematoxylin staining orderly. The sections were observed and imaged under a microscope (Leica, Germany). The images were scanned with Image J software to obtain the positive rate.

2.2.10. Detection of neutrophil viability

Neutrophil viability was tested by MTT colorimetric assay referring to the method of Gomez et al. (2013). Neutrophils (10,000 / well) were incubated in a 96-well plate until the preset target time. Then neutrophils were incubated for 4 h with 20 μl MTT (5 mg/mL) at 37 ℃. After formazan crystals were dissolved, the optical density (OD) was measured at 490 nm by enzyme labeled instrument (Thermo, China).

2.2.11. Detection of viability of A. fumigatus

Referring to the method of Gazendam et al. (2016) and Yekutiel et al. (2004), the viability of *A. fumigatus* was detected using XTT reduction assay. XTT and phenazine methosulfate (PMS) were dissolved in PBS to obtain final concentrations of 1 mg/mL XTT and 25 μM PMS. Hyphae were incubated with neutrophils (neutrophils 10,000/well, neutrophils: hyphae 10:1) in a 96-well plate. Neutrophils were lysed in H_2O/NaOH (pH of 11.0) before termination of the incubation. Subsequently, hyphae were incubated with 20 μl XTT/ PMS for 2 h. Then the supernatant in the well was carefully sucked and OD value was measured at 450 nm by enzyme labeled instrument (Thermo, China).

2.2.12. Flow cytometry

Neutrophils were stained using an Annexin V-FITC Apoptosis Detection Kit (sigma) according to the manufacturer's instructions. The specimens were analyzed using a BD Biosciences FACS Calibur flow cytometer.

2.3. Statistical analysis

All the statistical data were analyzed using SPSS 23.0 software. An unpaired Student *t* test was used when two groups were compared. Data was expressed as mean ± standard error (SE). A p value ≤ 0.05 was considered significant as indicated in the figures (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ in all figures).

3. Results

3.1. T2DM mice had a higher fungal load

The fungal load in lung tissues of mice (n = 5 per group) was respectively measured at 24, 48, 72, 120 and 168 h after fungi challenge. The fungal load of the control mice was high at 24–72 hours and decreased significantly at 120 h. The fungal load of T2DM mice remained at a high level all the time and continued to increase during the detection period, but the curve tended to be flat over time. The fungal load of T2DM mice was higher than that of the control mice at all time points, and the difference was statistically significant at 72 h ($P = 0.002$) and thereafter (Fig. 1a). The difference was further confirmed by lung histopathology. At 120 h, hyphae could still be seen in lung tissues of T2DM mice but were rare in the control mice (Fig. 1b).

3.2. T2DM mice had higher neutrophil counts

The neutrophil counts in BALF and circulating blood in both groups (n = 3 per group) were respectively measured before infection and at 24, 48, 72, 120 and 168 h after fungi challenge. Before infection, neutrophil counts in the two specimens of T2DM mice were higher than those in the control mice. After infection, neutrophil counts in the two specimens of each group rapidly increased, and at all time points, neutrophil counts of T2DM mice were higher than those of the control mice. Over time, neutrophil counts in the two specimens of the control mice decreased slowly, but continued to rise in T2DM mice (Fig. 1c and d). Because MPO is one of the markers of neutrophils, we measured MPO in lung tissues (n = 3 per group) at 48 h after infection by IHC. The positive rate of MPO in lung tissues of T2DM mice was statistically higher than that in the control mice (Fig. 1e and f).

3.3. Increased production of ROS and expression of NOX2 and MPO in lung tissues of T2DM mice

ROS in lung tissues at 48 h after fungi challenge (n = 3 per group) were detected by chemical fluorescence and DCFH-DA was used as a probe. ROS were statistically higher in T2DM mice than those in the control mice (Fig. 2a). Because ROS from neutrophils are mainly produced by NOX2 and MPO, NOX2 and MPO in lung tissues (n = 3 per group) were detected by IHC at 48 h after fungi challenge. The positive rates of MPO and NOX2 were higher in T2DM mice than those in the control mice (Fig. 1e, f, 2 b and c).

3.4. The increase of neutrophils and ROS was associated with more serious inflammation

At 72 h after infection, we conducted a histological examination of lung tissues and measured the levels of TNF-α and IL-1β in lung tissues (n = 3 per group). Compared with the control mice, T2DM mice had more severe congestion, hemorrhage and inflammatory cell infiltration, and more obvious alveolar septal edema (Fig. 2d). The level of IL-1β in T2DM mice was higher than that in the control mice, and the difference tended to be significant($P = 0.073$) (Fig. 2e). The level of TNF-α in T2DM mice was statistically higher than that in the control mice (Fig. 2f).

3.5. Decreased fungicidal ability of neutrophils in T2DM mice

Neutrophils and hyphae were co-incubated in vitro at a concentration ratio of 10:1 for 24 h and 48 h to observe the fungicidal ability of neutrophils (n = 3 per group). As expected, the fungicidal ability of neutrophils in T2DM mice was decreased when compared with that in the control mice, which was characterized by higher hyphae viability. As time passed, the difference in the fungicidal ability between the two groups gradually became obvious. The difference in hyphae viability between two groups was statistically significant at 48 h (Fig. 3a).

3.6. Increased intracellular ROS and decreased extracellular ROS in T2DM mice

The increase of ROS in lung tissues of T2DM mice may be related to the increase of neutrophil count. In order to find out how ROS produced by neutrophils changed, neutrophils and hyphae (n = 3 per group) were co-incubated for 6 h to detect ROS by DCFH-DA chemofluorescence assay. We found that ROS produced by neutrophils in T2DM mice were higher than those in the control mice (Fig. 3b). However, DCFH-DA assay only measures intracellular ROS, and the change of extracellular ROS was still not clear. Extracellular ROS were monitored by lucigenin Chemiluminescence assay (Maghzal et al., 2012) and thus considered as $O_2^{\cdot -}$. We found that extracellular $O_2^{\cdot -}$ was lower in T2DM mice than that in the control mice (Fig. 3c). In order to further clarify the differences of intracellular ROS and extracellular $O_2^{\cdot -}$ between the two groups, we repeated the above experiment without hyphae. We found that compared with the control mice, T2DM mice had increased intracellular ROS and decreased extracellular $O_2^{\cdot -}$ regardless of infection. After *A. fumigatus* infection, intracellular ROS increased and extracellular $O_2^{\cdot -}$ decreased in both groups (Fig. 3b and c).

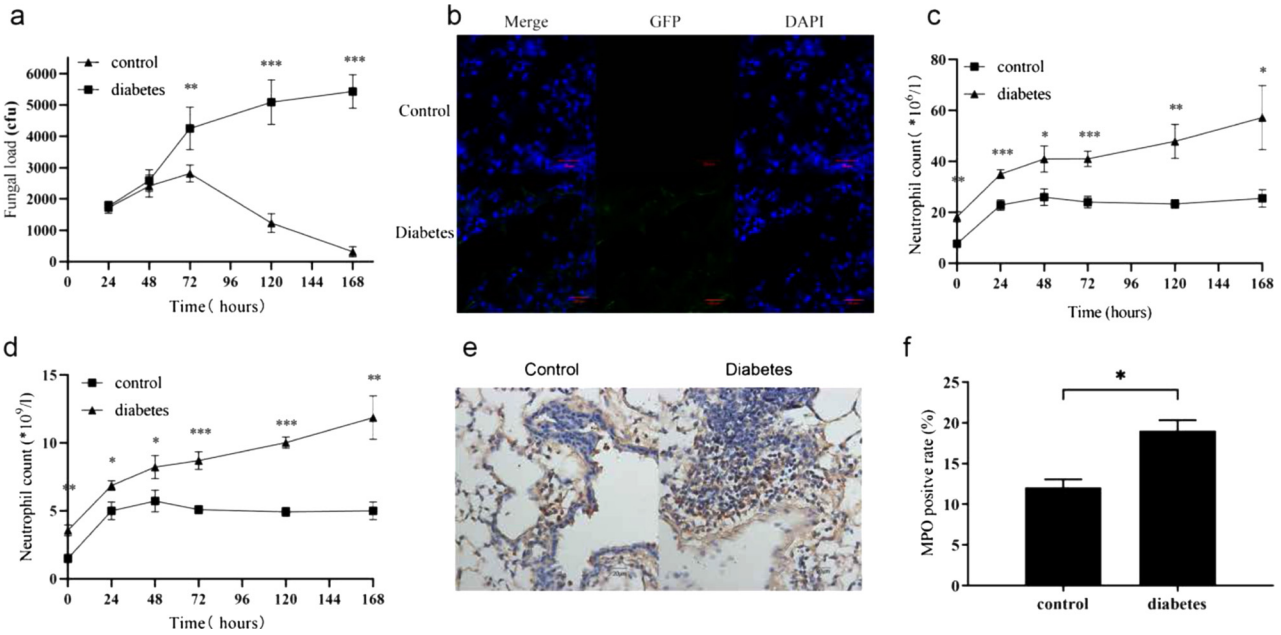

Fig. 1. Fungal loads in lung tissues and neutrophil counts in circulating blood, BALF and lung tissues. (a) Fungal loads in lung tissues at 24, 48, 72, 120 and 168 h after infection. (b) Fluorescence microscopy for location of *A. fumigatus* hyphae in lung tissues at 120 h after infection. GFP was expressed by *A. fumigatus* hyphae, and DAPI was used to stain the nucleus. (c)(d) Neutrophil counts in BALF and circulating blood at 24, 48, 72, 120 and 168 h after infection. (e)(f) Immunohistochemical analysis of MPO and MPO positive rate in lung tissue at 48 h after infection.

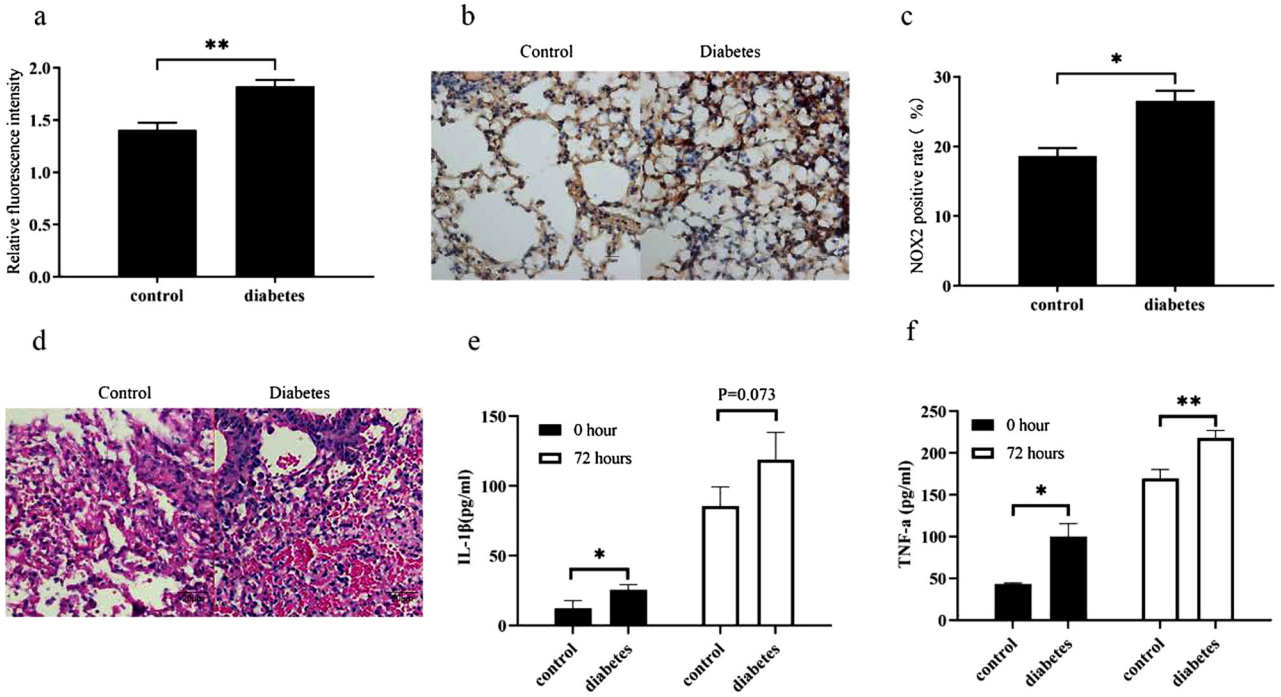

Fig. 2. ROS, NOX2 and inflammation in lung tissues. (a) ROS detected by chemical fluorescence at 48 h after infection. DCFH-DA was used as a ROS detection probe. Results are expressed in relative fluorescence intensity. (b)(c) Immunohistochemical analysis of NOX2 and NOX2 positive rate in lung tissue at 48 h after infection. (d) Lung histology at 72 h after infection. (e)(f) Expression of IL-1β and TNF-α in lung tissues at 0 h and 72 h after infection.

3.7. Increased intracellular ROS were related to increased cytokines

It had been previously confirmed that compared with the control mice, the IL-1β and TNF-α content in the lung tissue of T2DM mice was increased. In order to investigate whether the increase of IL-1β and TNF-α was related to neutrophils and the increase of intracellular ROS, the levels of IL-1β and TNF-α of the supernatants (n = 4 per group) were detected after co-incubation of neutrophils and hyphae in vitro. After a total of 24 h of incubation, the levels of IL-1β and TNF-α released by neutrophils in T2MD mice were higher than those in the control mice. After the addition of DPI (10 μM), the levels of IL-1β and TNF-α in both T2MD mice and the control mice decreased, and the effect was significant in the control mice, but not in T2MD mice. Moreover, the difference between two groups was still significant (Fig. 3d and e).

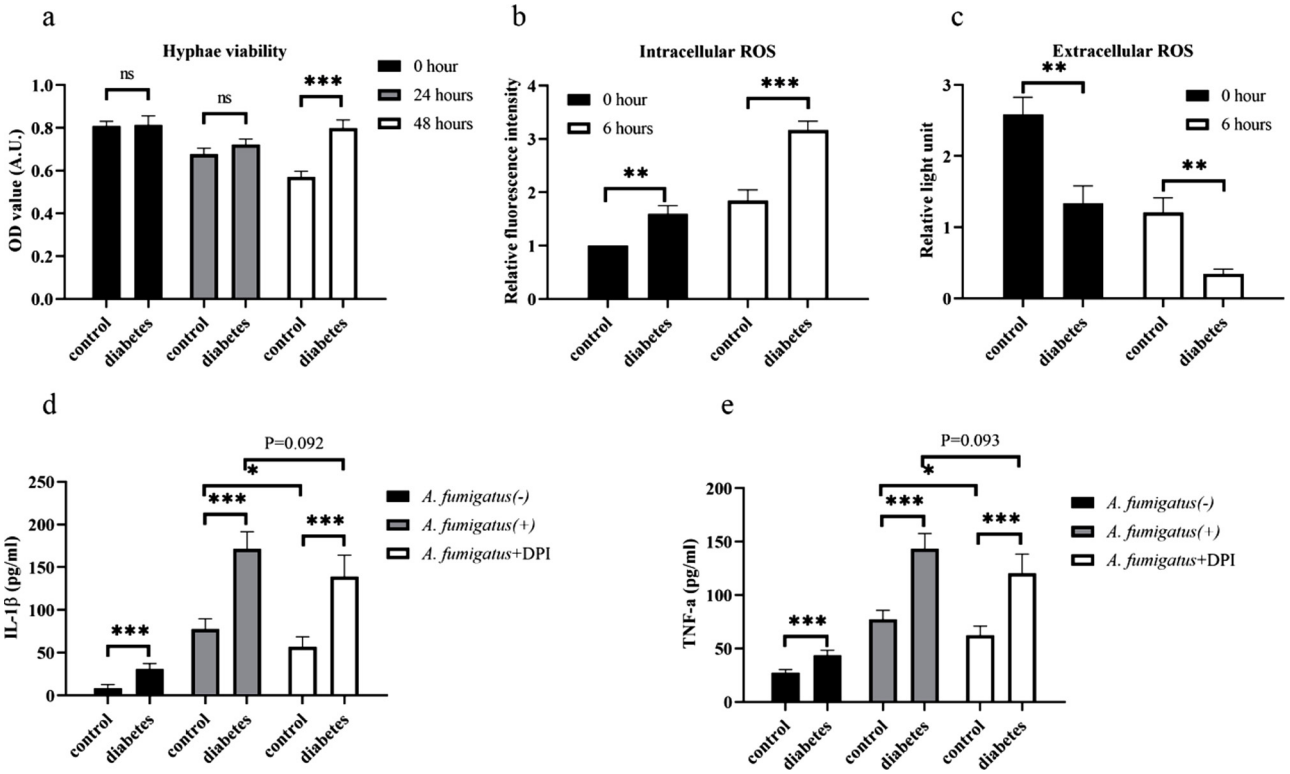

Fig. 3. Fungicidal ability, and the release of ROS and cytokines. (a) Hyphae viability detected by XTT assay at different time points. Results are expressed in OD value. (b)(c) Intracellular and extracellular ROS produced by neutrophils before co- incubation with hyphae and at 6 h after co-incubation with hyphae. Results are respectively expressed in relative fluorescence intensity and relative light unit. (d)(e) Levels of IL-1β and TNF-α in neutrophil supernatants after incubation for 24 h in vitro. *A. fumigatus* (-), neutrophils were incubated without hyphae; *A. fumigatus (+)*, neutrophils were incubated with hyphae; *A. fumigatus* + DPI, neutrophils were incubated with hyphae and DPI.

3.8. Increased intracellular ROS resulted in lower viability and more apoptosis

According to the above experimental results, the increase of intracellular ROS did not enhance the fungicidal ability of neutrophils in T2DM mice. In order to investigate whether excessive intracellular ROS caused damage to neutrophils in T2DM mice, the viability (n = 4 per group) and apoptosis (n = 3 per group) of neutrophils were detected after co-incubating neutrophils and hyphae in vitro. At 24 h after co-incubation, the neutrophil viability of T2DM mice was statistically lower than that of the control mice (Fig. 4a). At 48 h after co-incubation, the apoptosis rate of neutrophils in T2DM mice was statistically higher than that in the control mice (Fig. 4b and c). Since DPI can inhibit the generation of ROS by NADPH oxidase, we added DPI (10 μM) to the neutrophils suspension before adding hyphae and repeated the above test. Both T2DM mice and the control mice showed a statistical increase in neutrophil viability, and the difference between two groups was still statistically significant (Fig. 4a). The apoptotic rate of neutrophils in both T2DM and the control mice decreased, and the decrease of the control mice was not statistically significant, whereas T2DM mice had a statistically significant decrease. In addition, although the apoptotic rate of neutrophils in T2DM mice was still higher than that in the control mice after the addition of DPI, but the difference was reduced (Fig. 4b and c).

4. Discussion

Innate immunity plays a key role in the defense against *Aspergillus* infection. Neutrophils and macrophages are the main effector cells. Macrophages kill *Aspergillus* conidia, and neutrophils can kill both conidia and hyphae (Garth and Steele, 2017). The fungicidal activity of macrophages can be compensated by other white cells, but neutrophils are essential for the defense against *Aspergillus* infection (Mircescu et al., 2009). The inhibition of conidia germination only depends on the non-oxidative mechanism, but toxic ROS produced by NOX2 and MPO are very crucial for killing *Aspergillus* hyphae (Gazendam et al., 2016).

Diabetes affects the function of neutrophils (Szablewski and Sulima, 2017), and no matter in basal state or under stress, ROS production of neutrophils is abnormal in the diabetic patients (Hand et al., 2007; Wang et al., 2018). Oxidative stress induced by ROS plays a key role in the induction and progression of complications of T2DM. Under basal conditions neutrophils in patients with T2DM produce excessive ROS (Hand et al., 2007; Wang et al., 2018; Ridzuan et al., 2016), however, under stress the conclusions are not consistent. Some studies show that T2DM reduces the production of ROS (Marhoffer et al., 1992; Delamaire et al., 1997; de Souza et al., 2016; Frydrych et al., 2019), but other studies suggest that T2DM results in an increase in the generation of ROS (Hand et al., 2007; Ridzuan et al., 2016; Watanabe et al., 1993; Wong et al., 2002). Moreover, these conflicting conclusions have nothing to do with whether blood glucose is under control (Karima et al., 2005; Umsa-ard et al., 2015). This contradiction may be related to the difference of testing methods and experimental conditions, and also reflect that the ROS production of neutrophils in patients with T2DM is affected by many factors and has great variability. However, by far changes in ROS production of neutrophils in diabetic patients or animals with *Aspergillus* infection have not been studied.

At present, there is no animal model of diabetes combined with IA. In this study, T2DM mouse model was established by high-fat diet and STZ injection. Since non-immunodeficient mice will soon clear *A. fumigatus* conidia that have been inoculated via the airway, it is difficult to induce IA by inoculating the conidia only (Buskirk et al., 2014). Rohm et al. (2014) found that hyphae inoculation to mice with normal immune function could better simulate IA. Hyphae inhalation only is not the common way of infection in the real world, so we inoculated

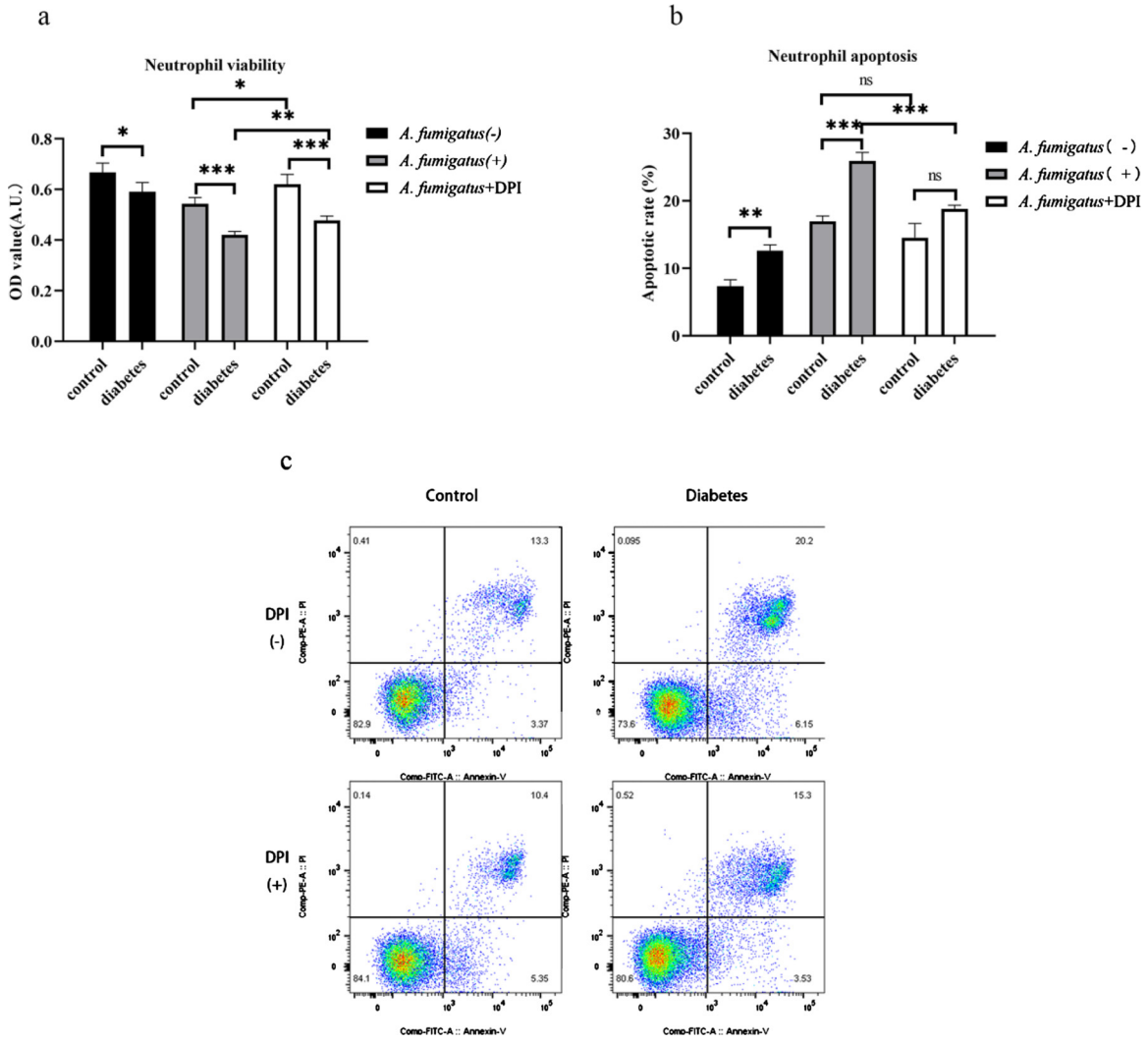

Fig. 4. Viability and apoptosis of neutrophils. (a) Neutrophil viability was detected by MTT assay at 24 h after incubation in vitro. Results are expressed in OD value. (b)(c) Apoptosis rate of neutrophils was detected by flow cytometry at 48 h after incubation in vitro. *A. fumigatus* (-), neutrophils were incubated without hyphae; *A. fumigatus (+)*, neutrophils were incubated with hyphae; *A. fumigatus* + DPI, neutrophils were incubated with hyphae and DPI.

mice with conidia before inoculating them with hyphae to simulate as much as possible the real infection way. We found that wild type C57BL/6 mice could remove pathogen quickly after inoculating, while T2DM mice had a continuous increase in pulmonary fungal load during the observation period of one week, indicating that T2DM mice were more susceptible than the control mice and could simulate IA better.

Using this model, we found that the neutrophil count from three different specimens (blood, BALF and lung tissue) and the content of ROS produced by neutrophils in lung tissues were higher in T2DM mice than those in the control mice. These changes did not lead to an improvement in fungicidal ability but were associated to more severe inflammation and tissue damage. Further in vitro studies showed that neutrophils of T2DM mice had decreased fungicidal ability and extracellular ROS and increased intracellular ROS compared with the control mice.

Intracellular and extracellular ROS production is regulated differently. Where ROS are released depends on where NOX2 is assembled and activated. NOX2 assembly on the plasma membrane results in the release of ROS to the extracellular milieu, whereas NOX2 assembly on an intracellular membrane would result in the release of ROS to the intracellular milieu (Bylund et al., 2010). Neutrophils can assemble NOX2 to release ROS in different locations by sensing the size of microorganisms. Warnatsch et al. (2017) found that neutrophils could engulf small-sized microbes to form phagosome and produce intracellular ROS, while neutrophils could only surround or attach to large-sized microbes (such as hyphae) which can't be engulfed and produce extracellular ROS. Gazendam et al. (2016) found that the killing of hyphae depends on extracellular ROS. However, Phan et al. (2018) found that neutrophils can use ROS to control bacterial infection at a distance in absence of phagocytosis. This finding sheds new light on the role of extracellular ROS, that is, the role of extracellular ROS may be more important than previously thought. In conclusion, in this study, the reduction of extracellular ROS may partly explain the decreased fungicidal ability.

Redox balance is finely regulated by the relative levels of oxidants (such as ROS) and antioxidants. Immune cell function is known to be influenced by the oxidant-antioxidant balance, and the accumulation of intracellular ROS triggers death receptor signals and plays a key role in the spontaneous apoptosis of neutrophils (Scheel-Toellner et al., 2004). In this study, increased intracellular ROS was associated with more severe neutrophil damage and tissue inflammation in T2DM mice.

Although interest in measuring products of the NOX protein family is great today, powerful analytical probes are limited. When using lucigenin as a probe, it is generally believed that lucigenin can reflect the level of extracellular $O_2^{\cdot -}$ due to its cell impermeability and selective for $O_2^{\cdot -}$, although $O_2^{\cdot -}$ production might be artificially overestimated

because of redox cycling phenomenon, in which the lucigenin radical can react with oxygen to generate $O_2^{\cdot -}$ (Liochev and Fridovich, 1997). Options for quantitation of intracellular specific ROS are very limited (Nauseef, 2014). DCFH-DA is one of the most commonly used probes for intracellular ROS. After DCFH-DA is taken up and hydrolyzed to DCFH by cells, oxidation of DCFH to fluorescent DCF is not directly achieved by $O_2^{\cdot -}$ or H_2O_2 (oxidation by H_2O_2 requires a catalyst), but can be by peroxyl, alkoxyl, $NO2^{\cdot}$, carbonate ($CO3^{\cdot -}$), $OH^{\cdot}$ radicals and peroxynitrite (Eruslanov and Kusmartsev, 2010). And like lucigenin, redox cycling may also occur when DCFH-DA probe is used (Rhee et al., 2010). Therefore, it is not appropriate to regard DCFH-DA probe as detecting a specific oxidizing species such as H_2O_2, but rather as a detector for a variety of oxidizing reactions that may be increased during intracellular oxidative stress (Eruslanov and Kusmartsev, 2010; Hempel et al., 1999). The complexity of DCFH oxidation also shows that it is difficult to draw conclusions about which aspect of neutrophil ROS chemistry actually works. Scheel-Toellner et al. (Scheel-Toellner et al., 2004) found that desferrioxamine, an inhibitor of hydroxyl radical generation via the Fenton reaction, can effectively delay neutrophil apoptosis. Based on this finding, we speculate that hydroxyl radical formed via the Fenton reaction might play an important role in intracellular oxidative stress.

Another problem is that DCFH-DA probe is not specific and does not allow subcellular localization, which means that it cannot distinguish between ROS derived from NOX2 and ROS derived from mitochondria. Mitochondria is one of main sources of intracellular ROS. Mitochondrial ROS can be measured by a mitochondria-targeted probe such as MitoSOX Red (Deshwal et al., 2018), but it is difficult to compare the results of these two different detection methods and mitochondrial ROS is not our target, so an inhibitor of NADPH oxidase, DPI, was used in this study and the effect of NOX2 was shown through DPI.

Neutrophils are an important source of cytokines. Some studies have found that intracellular ROS of neutrophils promote the release of IL-1β (Gabelloni et al., 2013), and the increase of ROS is accompanied by up-regulation of IL-1β expression (Ricci-Azevedo et al., 2018). But some other studies find that ROS inhibit the expression of IL-1β (Warnatsch et al., 2017). These findings suggest that the relationship between ROS and cytokines is complex and may be regulated by multiple factors and pathways. In this study, the effect of ROS on the expression of IL-1β and TNF-α was not significant, which may indicate that the role of ROS was not obvious, or the role may be masked by other factors.

5. Conclusions

Mechanisms for the increased risk of IA in diabetic patients are being explored. In this study, we found that there was a difference in the changes of intracellular and extracellular ROS of neutrophils in T2DM mice during IA. The increase of intracellular ROS caused injury to neutrophils and lung tissues, while the decrease of extracellular ROS ($O_2^{\cdot -}$) was related to the decreased fungicidal ability. The mechanism of imbalance of neutrophil intracellular and extracellular ROS in T2DM mice with IA is unclear, and research on this may help to prevent IA and improve the therapeutic effect of IA in T2DM patients.

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Author statement

Under the supervision and guidance of Professor Huang, Dr. Xu and Dr. Xia made significant contributions to the design and implementation of the work, data acquisition, analysis and interpretation, and drafted the manuscript. Professor Huang made strict revisions to the manuscript for important knowledge content and approved the revised version. All authors read and contributed to the manuscript. Professor Huang agreed to be responsible for all aspects of the work.

Declaration of Competing Interest

We confirm that there are no conflicts of interest.

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