Title

The Effect of Diluted Triple and Double Antibiotic Pastes on Dental Pulp Stem Cells and Established *Enterococcus faecalis* Biofilm.

AUTHORS

Alaa HA. Sabrah, [1] Ghaeth H. Yassen, [2] Wai-Ching. Liu, [2] W. Scott. Goebel, , [3] Richard L. Gregory, [4] Jeffrey A. Platt. [2]

[1] Department of Conservative Dentistry, The University of Jordan, Amman, Jordan.

[2] Department of Restorative Dentistry, Indiana University School of Dentistry, Indianapolis, IN, USA.

[3] Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN, USA.

[4] Department of oral biology, Indiana University School of Dentistry, Indianapolis, IN, USA.

CORRESPONDING AUTHOR

Alaa H.A. Sabrah

The University of Jordan

Department of Conservative Dentistry

Queen Rania Al-Abdullah street

Amman 11942 Jordan

Tel: +962-077-9862670; fax: +966-06-5355522.

E-mail address: sabralolo@yahoo.com

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ABSTRACT

Objectives: To investigate the effect of various dilutions of antibiotic medicaments used in endodontic regeneration on the survival of human dental pulp stem cells (DPSC) and to determine their antibacterial effect against established *Enterococcus faecalis* biofilm.

Materials and Methods: The cytotoxic and antibacterial effects of different triple (TAP) and double antibiotic (DAP) paste dilutions (0.125, 0.25, 0.5, 1, and 10 mg/ml) were tested against *Enterococcus faecalis* established biofilm and DPSC. Established bacterial biofilm were exposed to antibiotic dilutions for 3 days. Then, biofilms were collected, spiral plated and the numbers of bacterial colony forming units (CFU/mL) were determined. For the cytotoxic effect, lactate dehydrogenase activity assays (LDH) and cell viability assays (WST-1) were used to measure the percentage of DPSC cytotoxicity after 3 day treatment with the same antibiotic dilutions. A general linear mixed model was used for statistical analyses (α=0.05).

Results: All antibiotic dilutions significantly decreased the bacterial CFU/mL. For WST-1 assays, all antibiotic dilutions except 0.125 mg/ml significantly reduced the viability of DPSC. For LDH assays, the three lowest tested concentrations of DAP (0.5, 0.25, 0.125 mg/ml) and the two lowest concentrations of TAP (0.25 and 0.125 mg/ml) were non-toxic to DPSC.

Conclusions: All tested dilutions had an antibacterial effect against *Enterococcus faecalis*. However, 0.125 mg/ml of DAP and TAP showed a significant antibacterial effect with no cytotoxic effects on DPSC.

Clinical relevance: Using appropriate antibiotic concentrations of intracanal medicament during endodontic regeneration procedures is critical to disinfect root canal and decrease the adverse effects on stem cells.

KEYWORDS

Triple antibiotic paste, double antibiotic paste, *Enterococcus faecalis*, established biofilm, dental pulp stem cells.

Introduction

Endodontic regeneration techniques have received great attention in recent years. These techniques may offer a continuation of root development and improve the prognosis of necrotic immature teeth treatment [1-3]. Regeneration treatment protocol involves the use of an intracanal dressing with antibacterial properties followed by the induction of bleeding to create a matrix for the growth of new dento-pulpal tissue in the root canal space [4, 5]. Stem cells in the remaining vital pulp or apical papilla have been hypothesized to mediate tissue regeneration [1, 5].

Since its first use by Hoshino and his research group [6, 7], triple antibiotic paste (TAP), a combination of metronidazole, ciprofloxacin, and minocycline, is the most widely used intracanal medicament in endodontic regeneration [5, 8-10]. TAP use has been reported in 51% of published cases of endodontic regeneration [10]. To overcome the discoloration problem associated with the presence of minocycline in TAP, a combination of metronidazole and ciprofloxacin (DAP) was proposed [11] and used successfully in endodontic regeneration [12].

Due to the critical role of stem cells in endodontic regeneration, their viability in the presence of antibiotic pastes is essential for success. Recently, studies suggested that intracanal medicaments (TAP, DAP) are directly toxic to stem cells of apical papilla (SCAP), human periodontal ligament fibroblasts, human dental pulp cells, and apical papilla cells [13-16] and indirectly toxic to SCAP [17] in the concentrations currently used in endodontic regeneration. Most of the studies proposed that 0.1-2 mg/mL of TAP or DAP did not have any cytotoxic effect on dental pulp cells [13, 15, 16]. TAP and DAP were effective against newly formed bacterial biofilm at 0.03 and 0.01 mg/mL, respectively [18]. However, these relatively low concentrations of medicaments may not be effective against established bacterial biofilm. It is essential to explore the antibacterial effectiveness against established bacterial biofilm because of the critical involvement of biofilm in the etiology of periapical periodontitis [19]. It is also crucial to explore the optimum dilutions of antibiotic medicaments that have antibacterial effect against established bacterial biofilm without compromising the survival of human dental pulp stem cells. Therefore, the aim of this study was to explore the effect of various dilutions of antibiotic medicaments

used in endodontic regeneration on human dental pulp stem cells (DPSC) survival and to determine the antibiofilm effect of these dilutions against established biofilm of *Enterococcus faecalis* (*E. faecalis*).

MATERIALS AND METHODS

Antibacterial experiment

Bacterial Strain and Media

E. faecalis (ATCC 29212 strain) was initially grown on anaerobic blood agar plates (CDC, BioMerieux, Durham, NC USA). Then, bacteria cultures were grown in brain heart infusion broth supplemented with 5 g/l yeast extract (BHI-YE) at 37°C in an anaerobic environment using gas generating sachets (GaPak EZ, Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Antibiotic medicament preparation

For TAP, 100 mg of USP grade antibiotic powder compounded of equal portions of metronidazole, ciprofloxacin, and minocycline (CHAMPS Medical, San Antonio, TX, USA) was dissolved in 1 mL of sterile water (33.3 mg of each antibiotic/mL). For DAP (CHAMPS Medical), 100 mg of USP grade antibiotic powder compounded of equal portions of metronidazole and ciprofloxacin was dissolved in 1 mL of sterile water (50 mg of each antibiotic/mL). The stock solution of each antibiotic mixture was used to prepare the five experimental concentrations (10, 1, 0.5, and 0.25 and 0.125 mg/mL) by mixing the appropriate amount of the antibiotic solution with BHI-YE culture media for *E. faecalis* biofilm assay. The ranges of dilutions used in this study were selected according to the most recent available literature regarding both antibacterial and cytotoxic effect of these medicaments [7, 13, 15, 18]. The different dilutions were filter sterilized using a sterile 20 µm filter (Fisher Scientific, Newark, DE, USA). A control group of BHI-YE culture media with no antibiotics was also used.

Antibiotic effects on established bacterial biofilm

To determine the effectiveness of the different dilutions of TAP and DAP antibiotic medicaments on the established bacterial biofilm, 100 µl of an overnight *E. faecalis* culture (10^6 CFU/mL) in BHI-YE and 900 µl fresh BHI-YE were incubated for 3 days in independent wells of 12-well plates (Becton, Dickinson and Company). The culture media were not changed during the 3 days of biofilm cultivation in an attempt to

grow biofilm that is closer to the biofilm grows inside an infected root canal. A recent study found that frequent replacement of fresh media may lead to overgrowth of facultative cocci and formation of a biofilm that is untypical to root canal environment [20]. After the incubation time, planktonic bacterial in culture supernatants were removed from each well, and the remaining established biofilm was washed with saline solution (5mL over 60 minutes) and treated, in triplicate, with antibiotic dilutions in BHI-YE for 3 days. At the end of the incubation time, antibiotic dilutions were removed from each well, and the biofilm cells were gently washed with saline. The biofilm cells were collected by cell scrapers (Fisher Scientific), vortexed, diluted in sterile saline and spirally plated on tryptic soy agar plates (TSA). The plates were incubated for 48 h in a 5% CO_2 incubator at 37°C. Then, the number of colony forming units per volume (CFUs/mL) of each treated well was determined using an automated colony counter (Synbiosis, Inc., Frederick, MD, USA) and compared to the values from the control culture.

Cytotoxicity experiment

Human dental pulp stem cells (DPSC)

DPSC (Cook General BioTechnology LLC, Indianapolis, IN, USA) obtained from immature third molars were used in this study. The stem-like nature of these cells has been previously established utilizing flow cytometric analysis [21, 22]. The cells were cultured in DMEM media supplemented with 10% fetal bovine serum (Atlanta Biologicals Inc. Flowery Branch, GA, USA), 5% penicillin-streptomycin (Life Technologies Corporation, Grand Island, NY, USA) and 2% Amphotericin B (Life Technologies Corporation). Cells at passages between 3 and 5 were utilized in this experiment. Sub-confluent cells were detached from the culture plate with 0.05% trypsin and EDTA (Life Technologies Corporation). The evaluation of cytotoxic effects of the various TAP and DAP dilutions were determined using lactate dehydrogenase (LDH) activity and cell viability colorimetric assays utilizing water soluble tetrazolium salts (WST-1).

LDH assays

Sub-confluent DPSC were seeded into a 96-well plate (10,000 cells/well) and incubated for 24 hours in DMEM supplemented with 10% fetal bovine serum. After 24 hours, the old media was removed,

and the cells were incubated with serum free DMEM in the presence of different concentrations of TAP or DAP mixed with DMEM culture media. Additionally, DPSC incubated without any medicament in serum free DMEM were used as a negative control. After 3 days, the media were collected and utilized for the LDH assays. The levels of LDH released by the cells into the media due to membrane damage were determined using the Pierce™ LDH cytotoxicity assay kit (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA). Briefly, 50 µl of DPSCs culture media were transferred into another 96-well plate. Then, 50 µl of LDH assay mix was prepared according to the manufacturer's protocol, added and mixed in each well, followed by incubation for 30 minutes at room temperature. A spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) was used to quantitatively measure the colorimetric change at a wavelength of 490 nm. Positive control wells (Maximum LDH release) were also used by incubating DPSCs with the lysis solution provided by the manufacturer for 45 minutes (100 µl of serum-free DMEM and 10 µl lysis solution). The following equation was used to calculate the percentage of cytotoxicity due to LDH release from the treated cells relative to the untreated control:

Cytotoxicity (%) = (experimental value – negative control) / (positive control – negative control) ×100.

The positive control cytotoxicity percentage was considered to be 100%, while the percentage cytotoxicity of the negative control was 0%.

WST-1 assays

After collecting the media for LDH assays and washing the cells with phosphate buffer solution, the DPSCs were incubated with 10 µl WST-1 and 90 µl serum-free DMEM for 4 h in a humidified atmosphere at 37°C and 5% CO_2. After that, a spectrophotometer was used to measure the optical absorbance of each well at 450 nm. Negative controls consisting of 10 µl WST-1 incubated with 90 µl serum-free DMEM without the presence of cells were used as a blank. The following equation was used to calculate the percentage of the absorbance values of the antibiotic-treated cells at each concentration in comparison to the absorbance values of the untreated cells.

The percentage of cell viability (%) = (absorbance value of the treated cells /absorbance value of untreated cells) ×100. The percentage cell viability of the untreated cells was considered 100%.

Statistical Analysis

Each experimental treatment was conducted in triplicate and repeated individually three times. Data were analyzed using a linear mixed model (SAS software 9.4, SAS Institute, NC, USA) to test the effect of treatment on both bacteria and DPSC. The untreated control groups in both experiments were used as reference groups and they were removed from the model. The significance level was set at 0.05.

Results

A significant decrease in the log CFU/mL of *E. faecalis* bacteria was noticed with all dilutions of TAP and DAP compared with the untreated control ($P<0.0001$). For TAP, a gradual and significant reduction in antibacterial activity was observed between different dilutions ($P=0.003$-0.0001) (Figure 1). However, 0.125 and 0.25 mg/mL concentrations of TAP were not significantly different from each other. For DAP, a gradual and significant reduction in antibacterial activity was observed between different dilutions ($P=0.03$-0.0001) (Figure 1). However, 0.125, 0.25, and 0.5 mg/mL concentrations of DAP were not significantly different from each other. The log reductions in bacterial CFU/mL were 8.4, 6.5, 3.3, 1.7, and 2.3 for 10, 1, 0.5, 0.25, and 0.125 mg/ml TAP, respectively. The log reductions in bacterial CFU/mL were 8.4, 6.8, 3.4, 3.9, and 2.9 for 10, 1, 0.5, 0.25, and 0.125 mg/ml DAP, respectively.

For the LDH cytotoxic assays, the two highest tested dilutions of DAP (10 and 1 mg/mL) produced a significant cytotoxic effect on DPSC compared to the untreated control ($P < 0.001$) (Figure 2). For TAP, the three highest tested dilutions (10, 1, and 0.5 mg/mL) produced a significant cytotoxic effect on DPSC compared to the untreated control ($P =0.006$- 0.0001). For WST-1 assays, all dilutions of TAP and DAP significantly impaired the viability of DPSC ($P < 0.0001$) except 0.125 mg/mL (Figure 3).On the other hand, 0.125 mg/mL of TAP produced a significant enhancement in DPSC viability compared to untreated control ($P < 0.0001$). Images of DPSC in the presence of different dilutions of TAP and DAP (0.25-10 mg/ml) are presented in Figure 4.

Discussion

One of the distinguishing characteristics of endodontic microorganisms is their ability to form biofilms. Indeed, apical periodontitis has been classified as a biofilm-induced disease [19]. The use of minimum or no instrumentation during endodontic regeneration requires an effective chemical challenge to eradicate the endodontic pathogens. However, a root canal in an infected immature tooth has a large, blunder buss apical foramen, which may cause the disinfecting agent to pass through the open apex and induce a cytotoxic effect to the stem cells present in the periapical area. Furthermore, remaining vital pulp in immature teeth may be in direct contact with the disinfecting agent. Therefore, it is essential to achieve a balance between the antibacterial effect and the cytotoxic effect of any suggested intracanal medicament.

In this study, both TAP and DAP at a concentration of 10 mg/mL were able to eradicate all the established biofilm of *E. faecalis* after 3 days. All other concentrations of both antibiotic pastes significantly reduced the established *E. faecalis* biofilm but did not completely eradicate it. The antibiotic concentration required to completely eradicate the biofilm in this study (10 mg/mL) was higher than the concentrations reported in previous studies [6, 7], which ranged from 0.075 to 1.5 mg/mL of TAP. However, an agar diffusion test rather than bacterial biofilm was used in one study [6], and early biofilm formation was measured in the other study [7]. A recent study reported that TAP and DAP were able to significantly reduce new biofilm formation of *E. faecalis* and *Porphyromonas gingivalis* at concentrations of 0.01 mg/mL for DAP and 0.03 mg/mL for TAP using a microtiter plate method [18]. However, the previous study tested the antibacterial effects against early biofilm formation rather than established biofilm. To the best of our knowledge, this is the first report that investigates the antibacterial effect of diluted antibiotic medicaments on established bacterial biofilm which is more representative of the actual clinical situation. The established biofilm in this study was grown for only 3 days. However, bacterial established biofilm in the root canal might be formed in a longer period of time before patient seeks treatment. Future researches should investigate the antibacterial concentrations needed to eradicate

older established bacterial biofilm. *E. faecalis* was used as *in-vitro* model because it is a commonly isolated microorganism from persistent endodontic infections [23], it poses various survivals and virulence factors that enables it to resist nutritional deprivation [24] and hence resist treatment. Additionally, *E. faecalis* is described as a high biofilm producer [25, 26]. Future studies are needed to identify antibiotic concentrations that are effective against bacterial biofilm taken from immature teeth with infected root canals.

A concentration of 1 mg/ml of TAP or DAP was also effective in eradicating more than 99.9999 % of *E. faecalis* established biofilm (more than 6 unites log reduction in bacterial CFU/mL). A material can be considered effective in eradicating the bacterial biofilm when it can produce a reduction of more than 5 logarithmic units in the log CFU/mL (99.999 % reduction) compared to untreated control [27-29]. The lowest three concentrations of both TAP and DAP (0.125-0.5 mg/mL) did not produce a reduction of 5 logarithmic units in the log CFU/mL compared to untreated control. However, the antibacterial effect measured in this study was accomplished after only 3 days of antibiotic application. The use of these concentrations in clinical situations might be more efficient since the application time of intracanal medicaments usually range between 1 to 4 weeks [10]. A material can be considered bacteriocidal when it can produce a reduction of more than 3 logarithmic units in the log CFU/mL (99.9 % reduction in bacterial biofilm). A recent study has reported that the minimum bactericidal concentration of TAP and DAP against *E. faecalis* were 0.3, and 0.14 mg/ml, respectively [18]. This is in agreement with our results which show that TAP concentrations lower than 0.3 mg/ml (0.25, and 0.125 mg/ml) did not produce 3 log reductions in CFU/mL. Similarly, 0.125 mg/ml of DAP in our study did not produce 3 log reductions in CFU/mL because it falls under the minimum bactericidal concentration identified previously [18].

Different substrates have been used to develop bacterial biofilms in endodontics such as dentin, polystyrene culture plates and hydroxyapatite disks [30]. Polystyrene culture plates, a popular substrate in *in-vitro* endodontic experiments [18, 25, 31], was used in the current study. This substrate was suggested to offer rapid retrieval and quantification of bacterial biofilm [30, 31]. On the other hand, the use of

dentin substrate in antibiofilm studies is closer to the clinical situation. However, a relatively recent study suggested similar pattern of antibiofilm effect against *E. faecalis* developed in both polystyrene plates and root canal dentine [32].

For LDH cytotoxicity, the three lowest tested concentrations of DAP (0.125, 0.25 and 0.5 mg/mL) and the two lowest concentrations of TAP (0.125 and 0.25 mg/mL) were not toxic to DPSCs. A recent study reported that 0.3-2.5 mg/mL of TAP and 0.5-1 mg/mL of DAP were not toxic to dental pulp fibroblasts using LDH assays [15]. The difference in detected cytotoxic concentrations using LDH assays between the current and previous study could be explained by the different type of cells used (pulp stem cells versus pulp fibroblasts).

For WST-1 assays, only 0.125 mg/mL of both antibiotic mixtures was not toxic to DPSCs. This is in agreement with previously reported concentrations of both DAP and TAP (0.1 mg/mL) that did not have any toxic effects against stem cells of dental papilla (SCAP) [13]. In this study, DPSC were in direct contact with the antibiotic medicament. The concentration used in our study (0.125-10 mg/mL) fall under the concentrations reported previously for saturated solution of TAP and DAP (100, and 50 mg/mL, respectively) [18]. Therefore, direct cytotoxicity from undissolved medicament is not expected. Our results are in agreement with the results reported by Ruperal et al., [13] which demonstrate that the safest concentration of DAP or TAP on stem cells is 0.1 mg/mL. Regardless of the different methods to detect cytotoxicity (colorimetric assays vs cell counting), different mesenchymal stem cells (DPSC and SCAP) seems to behave similarly in the presence of antibiotic medicaments compared to differentiated cells (i.e., dental pulp cells) which have been reported to withstand higher concentrations of TAP and DAP (2 and 0.3 mg/mL, respectively) [15].

In the current study, cytotoxicity was detected earlier using WST-1 assays compared to LDH assays. This could be explained by the capability of the WST-1 to detect change in mitochondrial metabolic activities inside the cells that may precede the cell membrane destruction detected by LDH assays. Previous studies that used pulp fibroblasts or other types of cells have also reported earlier

detection of cytotoxicty using WST-1 assays compared to LDH assays [15, 33, 34]. Various cytotoxic assays may give different cytotoxic results depending on the measured physiological endpoint. Therefore, any clinical extrapolation of a detected cytotoxic concentration should be based on more than a single cytotoxic assay.

According to both antibacterial and cytotoxicity assays, a concentration of 0.125 mg/mL of both TAP and DAP was able to significantly reduce the bacterial biofilm without causing any cytotoxicity to DPSC. The American Association of Endodontists (AAE) has recently recommended the use of antibiotic medicaments in endodontic regeneration in concentrations ranging from 0.01 to 0.1 mg/mL [10, 35]. However, this concentration might not be effective against established bacterial biofilm. Additionally, no clinical studies have reported the use of these recommended diluted concentrations of antibiotics. Furthermore, a full concentration of TAP (1000 mg/mL) is still used in recently published clinical studies [36, 37]. One of the challenges associated with the use of diluted antibiotic concentrations in immature infected teeth is that they are in liquid form and do not have a pasty consistency. Incorporation of the diluted antibiotics within a vehicle may be a good option to efficiently deliver the intended concentration of antibiotics into the root canal. Another approach to deliver these low antibiotic concentrations into the root canal could be through the use of a three-dimensional scaffold system. Recent studies have proposed the use of a bio-resorbable antibiotic-loaded scaffold to disinfect the root canal space in endodontic regeneration [38, 39]

Conclusions

Within the limitation of this *in vitro* study, only 0.125 mg/ml of TAP and DAP did not negatively affect the viability of DPSC. However, this concentration was not enough to completely eradicated established biofilm of *E. faecalis*. Antibiotic intracanal medicament with a concentration that can offer antibiotic effect against endodontic pathogens with minimum adverse effect against pulp stem cells should be considered during endodontic regeneration procedures.

Conflict of interest

WSG is consulting medical director of Cook General BioTechnology, LLC. The other authors declare no potential conflicts of interest.

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Captions:

Figure 1: The mean log CFU/mL of *E. faecalis* after incubation with different TAP and DAP antibiotic medicament dilutions for 72 h. The different groups are color coded in ascending order representing the increase in the mean log CFU/mL. Different letters represent statistically significant differences.

Figure 2: The percent cytotoxicity to DPSC in the presence of different TAP and DAP antibiotic dilutions for 72 h using LDH assay compared to the negative control set at 0% and the positive control set at 100% cytotoxicity. * Significantly higher than the control. ** Significantly lower than the control.

Figure 3: The percent viability of DPSC in the presence of different TAP and DAP antibiotic dilutions for 72 h using WST-1 assay compared to a negative control set at 100% and a positive control set at 0% viability. * Significantly higher than the control. ** Significantly lower than the control.

Figure 4: Images of DPSC in the presence of different TAP and DAP antibiotic dilutions for 72 h. (A) control group. (B) The number of DPSC decreased in the presence of 0.25 mg/ml DAP. (C, D) The decrease in number and change in shape of DPSC in the presence of 0.5 and 1 mg/ml DAP, respectively. (E) Complete loss of viability and cell membrane integrity of DPSC in the presence of 10 mg/ml DAP. (F) The number of DPSC decreased in the presence of 0.25 mg/ml TAP. (G, H) The decrease in number and change in shape of DPSC in the presence of 0.5 and 1 mg/ml TAP, respectively. (I) Complete loss of viability and cell membrane integrity of DPSC in the presence of 10 mg/ml TAP.

Figure 1
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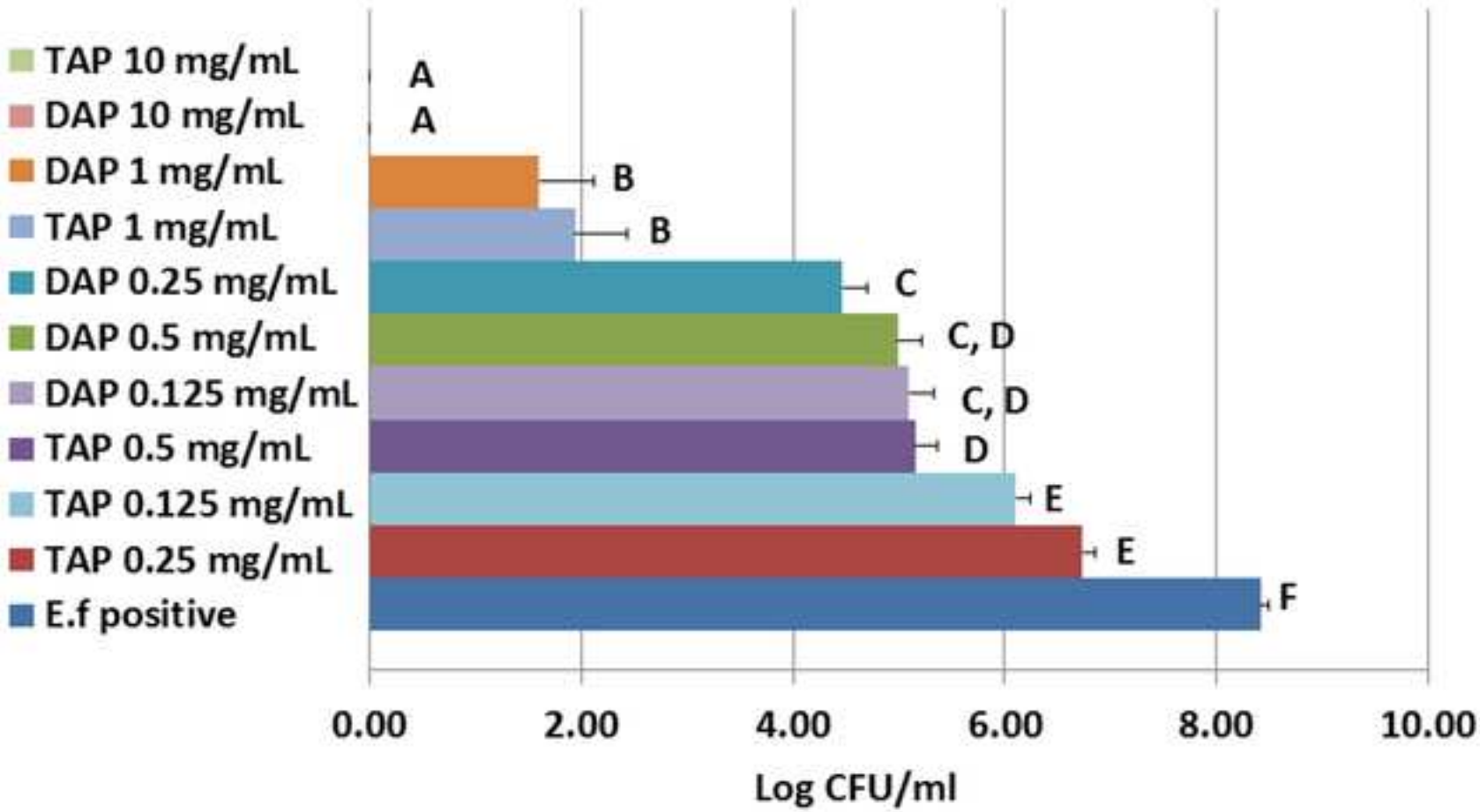

Figure 2
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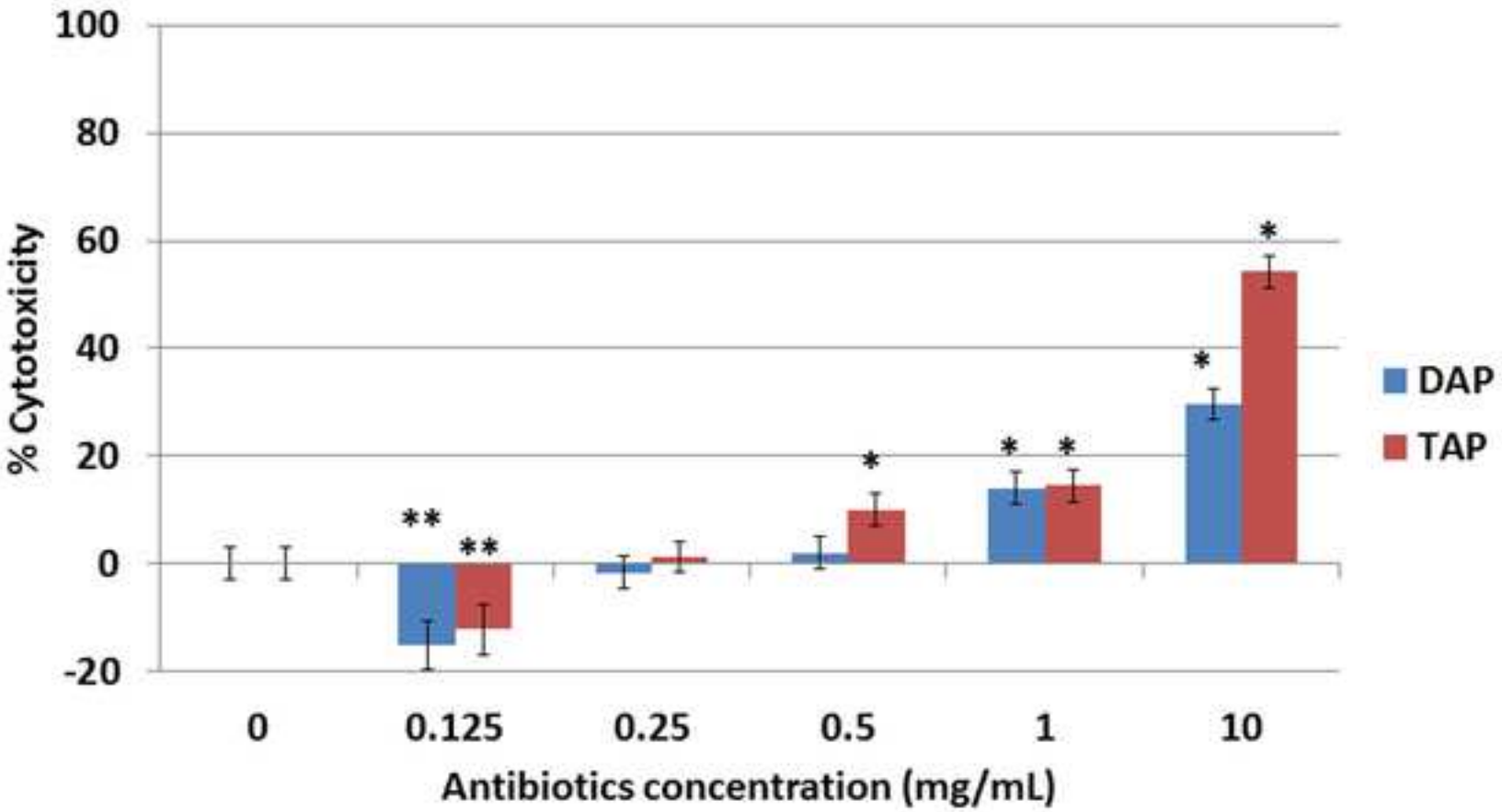

Figure 3
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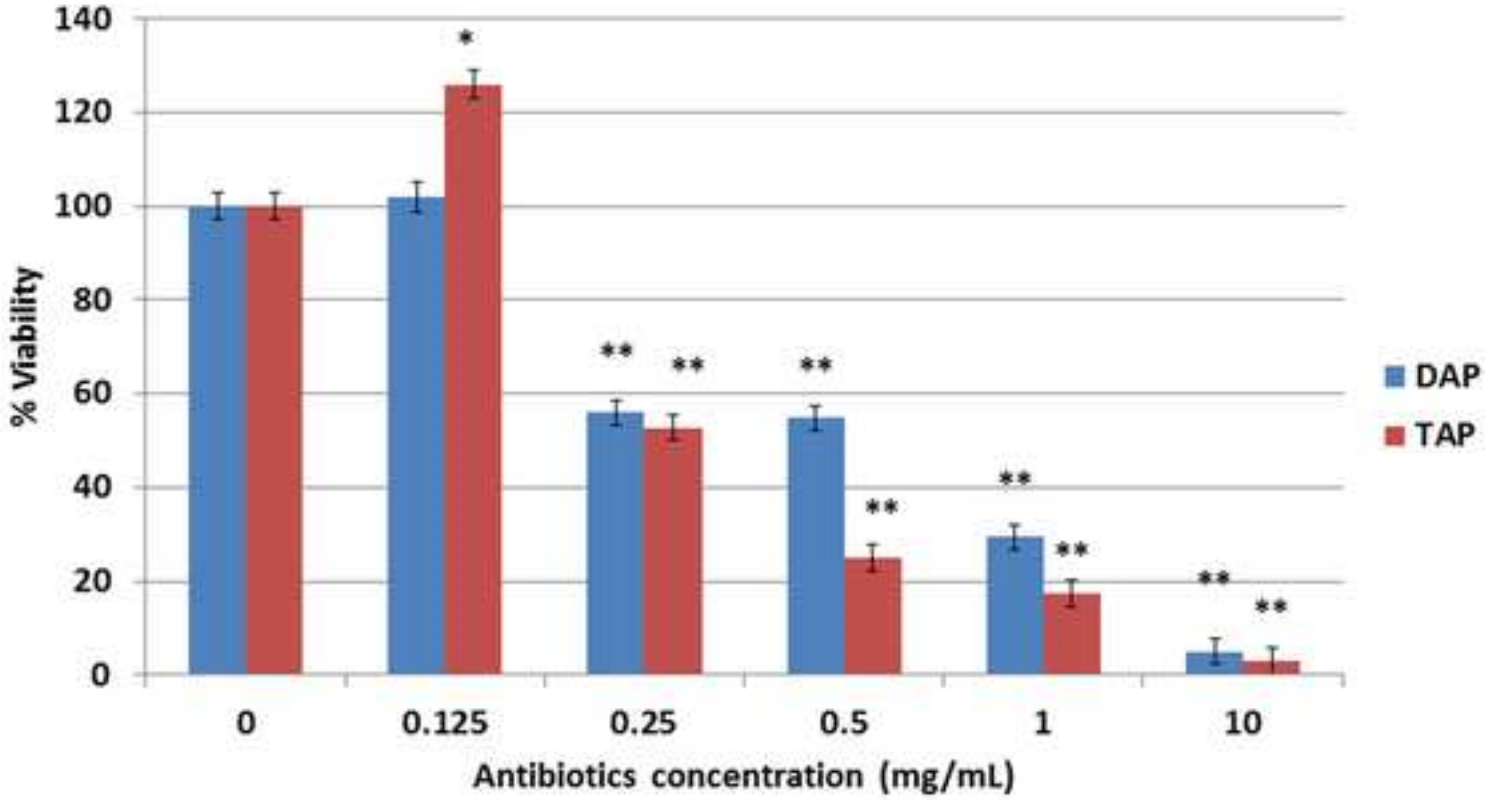

Figure 4A
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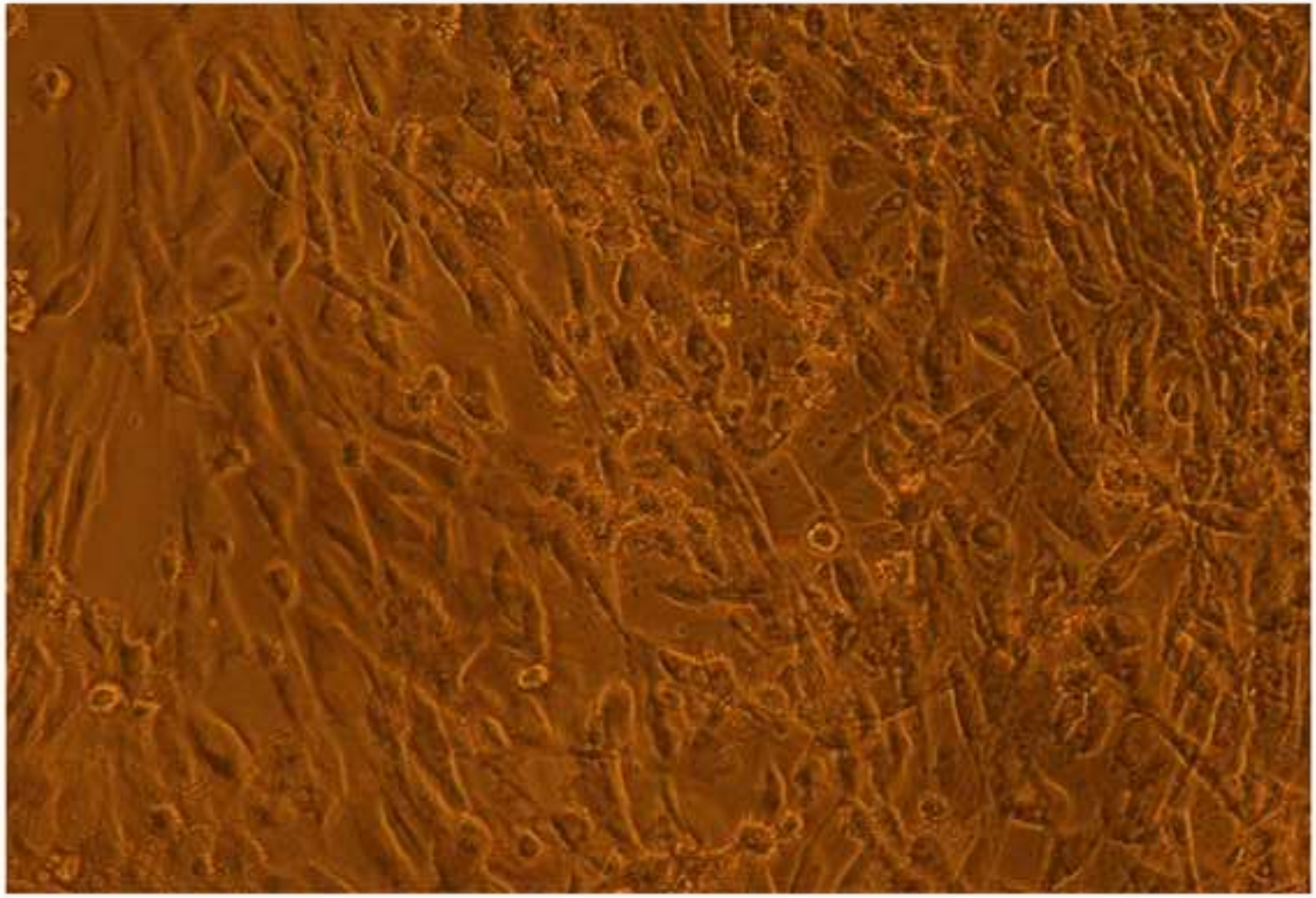

Figure 4B
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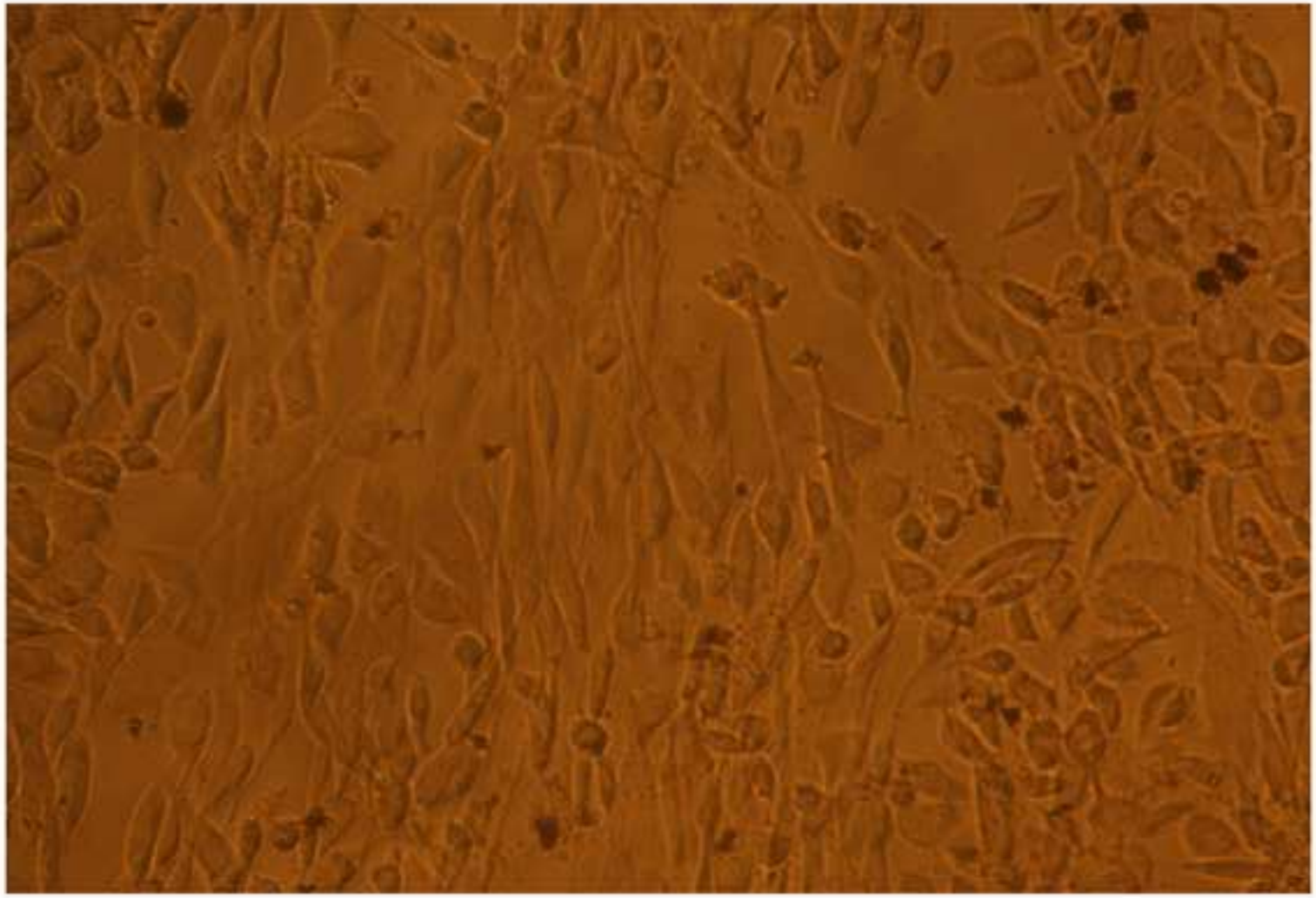

Figure 4C
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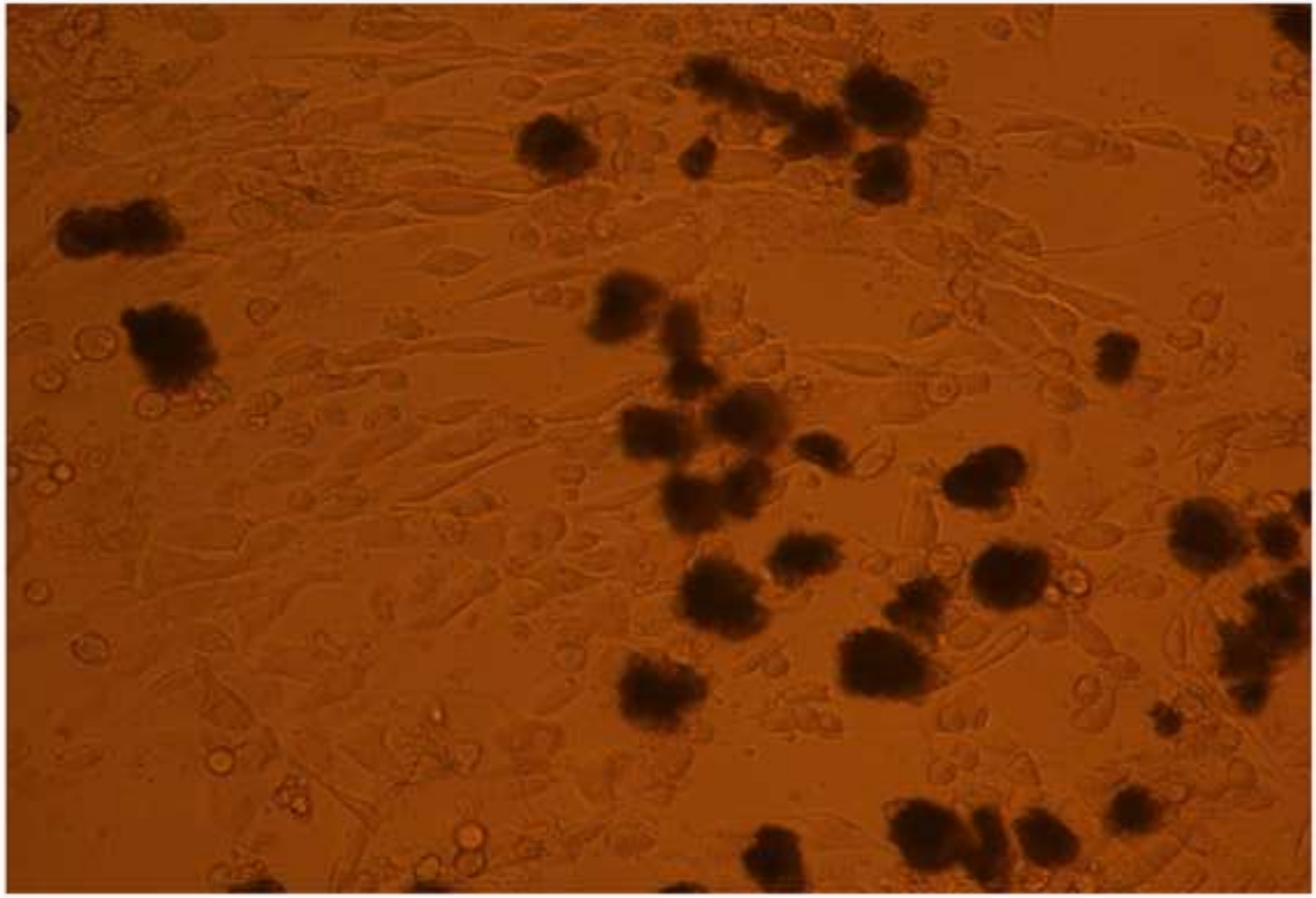

Figure 4D
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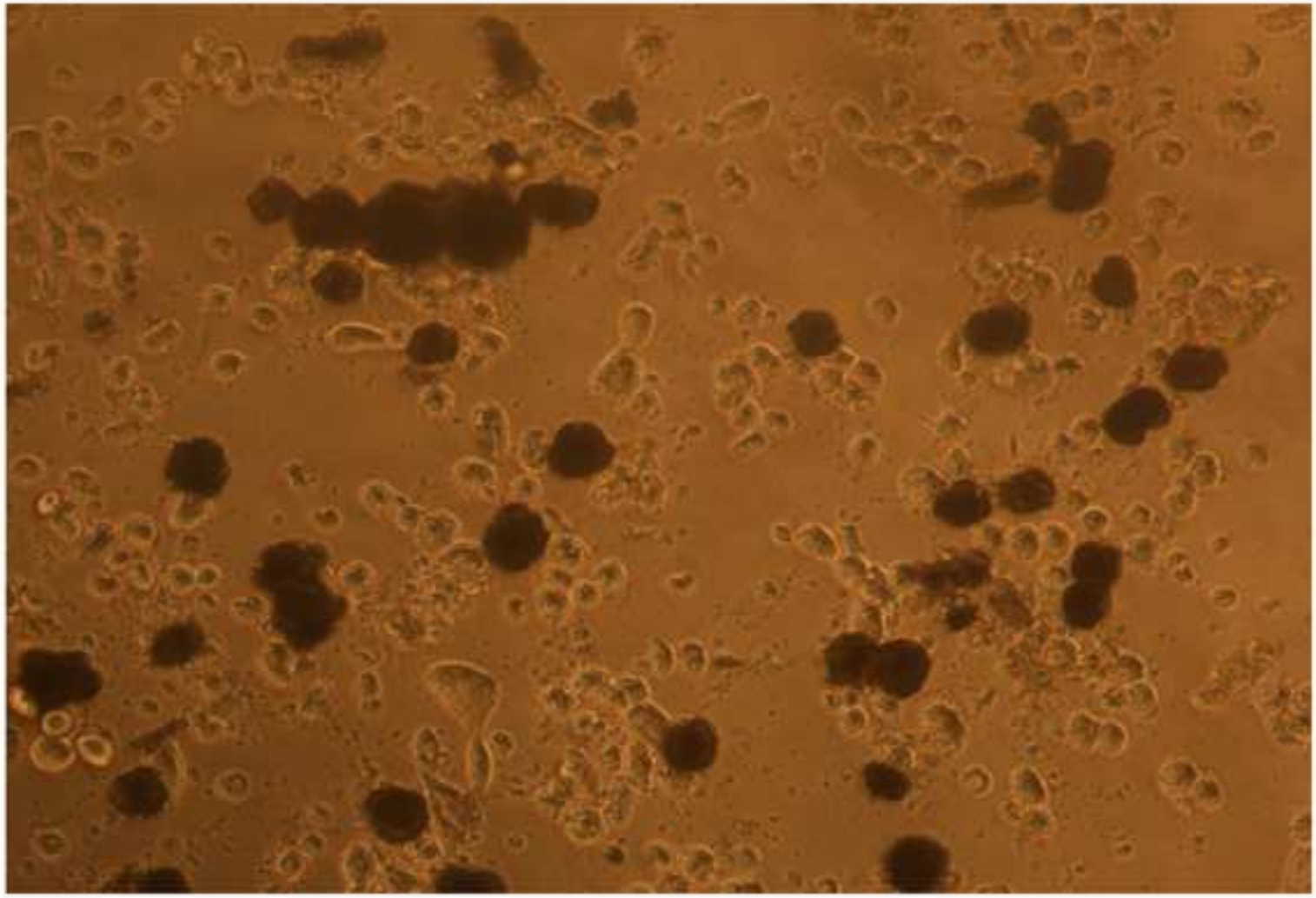

Figure 4E
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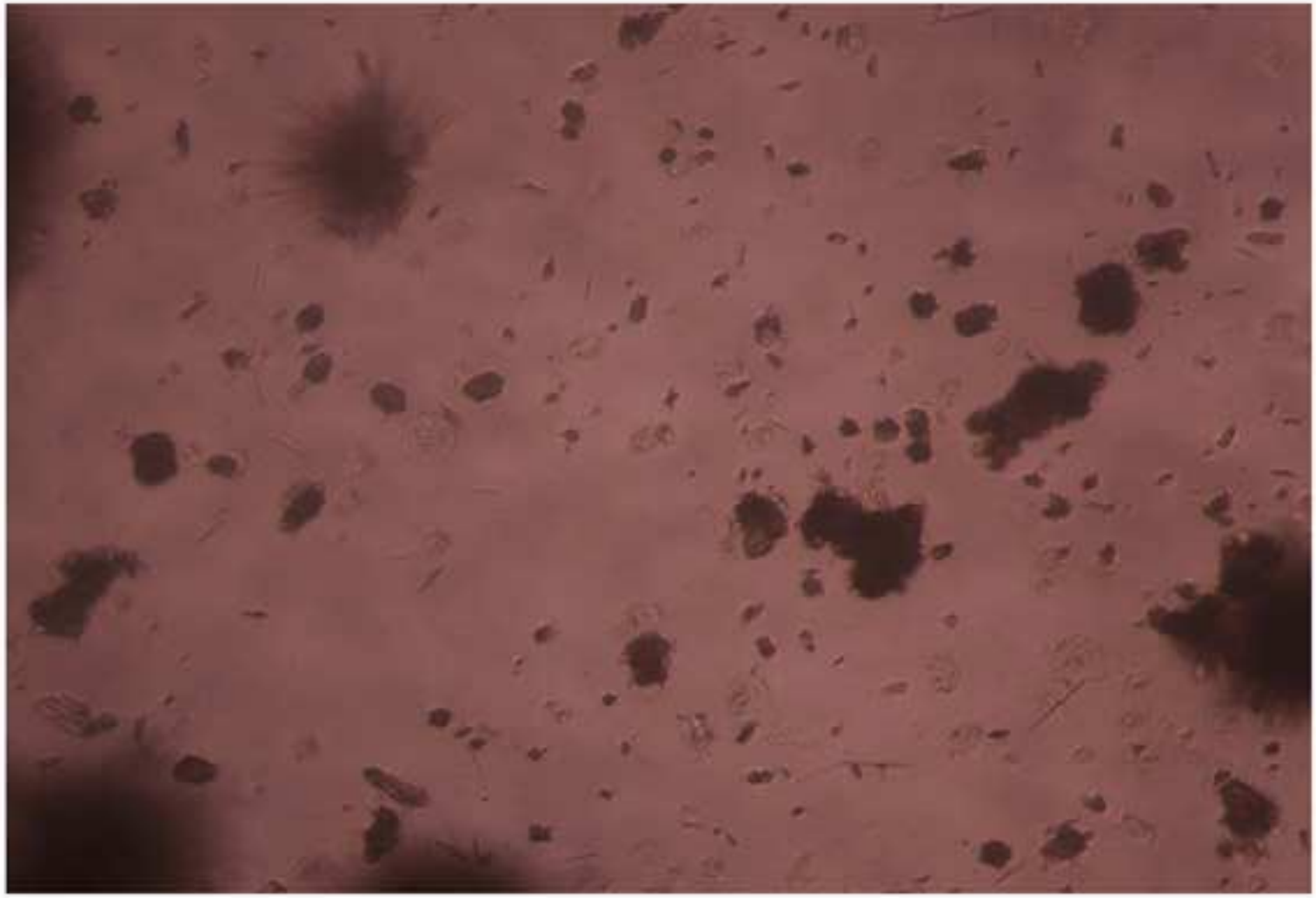

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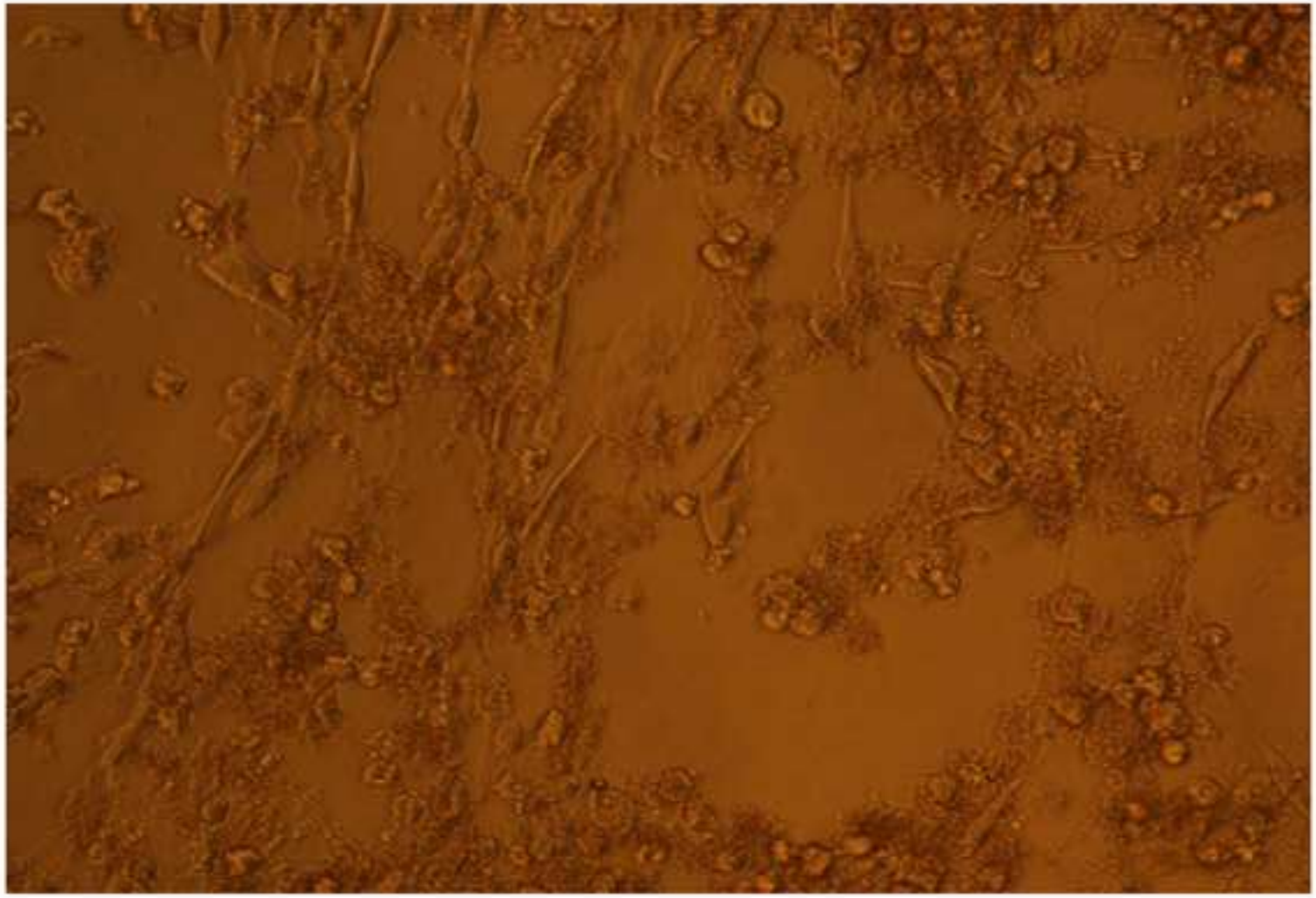

Figure 4G
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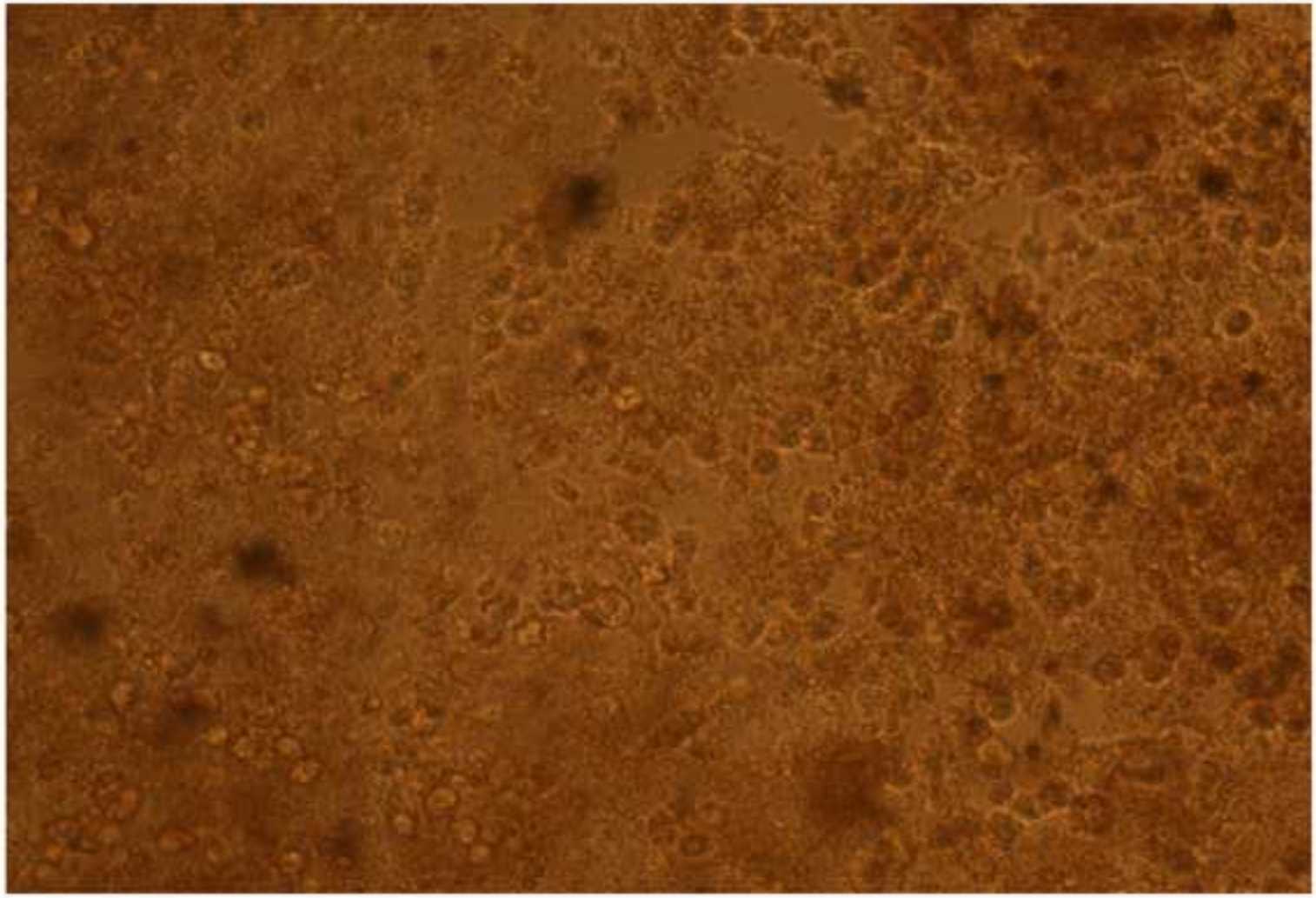

Figure 4H
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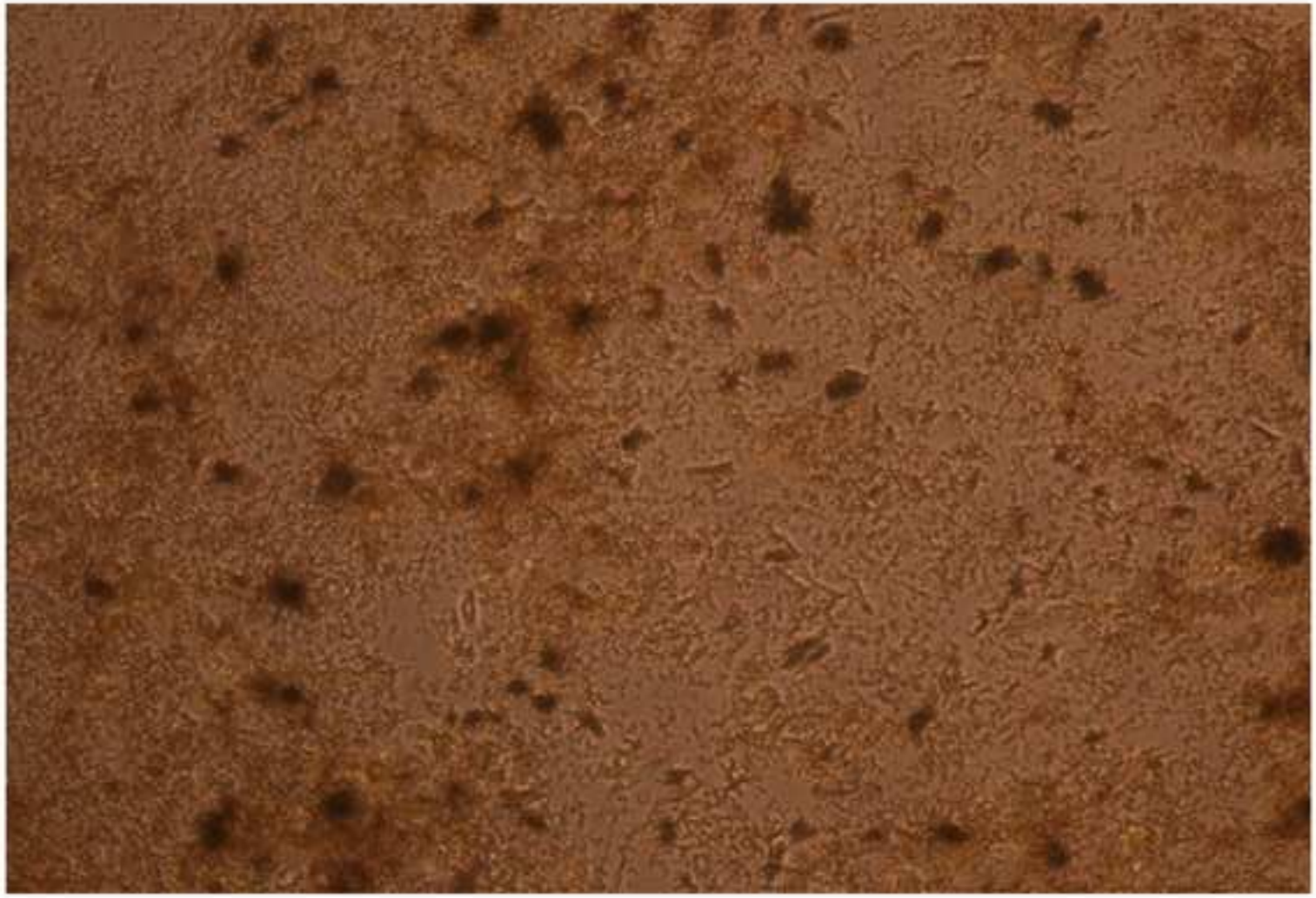

Figure 4I
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