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Determination of cytotoxicity of traditional Chinese medicine herbs, *Rhizoma coptidis*,*Radix scutellariae*, and *Cortex phellodendri*, by three methods

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ABSTRACT

Background: Many herbs are used in traditional Chinese medicine TCM) for treatment of infections but their properties, in particular, their effects on normal cells have received little attention. This study investigated the cytotoxic properties of three TCM herbs with potential use in prevention and treatment of ocular infections, including *Acanthamoeba keratitis*.
Method: The study investigated cytotoxic effects of the herbal extracts of *Rhizoma coptidis*, *Radix scutellariae*, and *Cortex phellodendri* on human corneal epithelial cells using trypan blue staining, MTT production, and flow cytometry. Differences between herbs were determined using repeated measures one-way analysis of variance, followed by paired *t*-tests where appropriate.
Results: These three herbs appeared to lack cytotoxicity when tested with trypan blue and MTT, but flow cytometry revealed that *R. coptidis* led to cell membrane damage.
Conclusion: Lack of cytotoxicity of *R. scutellariae* and *C. phellodendri* extracts suggest that these are potentially suitable for use in ocular preparations. Only flow cytometry was able to accurately predict cytotoxic effects of extracts of TCM herbs on HCEC, demonstrating the importance of using a sensitive method of detection of cytotoxicity.

1. Background

Although traditional Chinese medicine (TCM) has been used for the treatment of infections for a considerable period of time, investigations into their efficacies and active agents have only commenced very recently to provide for evidence-based use of these agents in a western medicine context. Of the herbs used for microbial infections, three have received wide attention: *Rhizoma coptidis*, *Radix scutellariae*, and *Cortex phellodendri*. *R. coptidis* was found to contain quaternary protoberberine alkaloid (QPA), quaternary aporphine alkaloid, flavonoids, tetracyclic triterpenes, and organic acids [1]. However, the concentration of these compounds varies, depending on the growth conditions, storage, and processing of the herb [2]. The herbal extract has been shown to be effective against *Staphylococcus aureus* [3], including methicillin resistant *S. aureus* [4] and *Candida* species [3]. In addition, it was found to reduce bacterial cell adhesion [5]. *R. coptidis* has also shown effects against protozoa including *Entamoeba histolytica*, *Giardia lamblia*, and *Trichomonas vaginalis* [6] and enhances the effects of antimalarial drugs in chloroquine-resistant strains [7]. As this agent has a wide antimicrobial spectrum, including protozoa, it may well be a useful agent in the prevention and treatment of ocular infections, including *Acanthamoeba* keratitis (AK). AK is a serious ocular infection, associated with the use of contact lenses and exposure to tap water [8]. Currently, treatment for *Acanthamoeba* infection requires hourly instillation of disinfecting agents, including chlorhexidine and polyhexamethylene biguanide (PHMB). Although this has been shown to be effective [9] the frequency of application can be problematic. Multipurpose solutions have variable effects on the viability of *Acanthamoeba* [10–12].

The major components of *R. scutellariae* are flavonoids, including baicalin, baicalein, wogonin, and wogonoside [13]. These agents have been shown to inhibit bacteria as well as yeast and other fungi [14]. They have also been shown to be effective against several viruses including HIV [15], hepatitis virus [16], and influenza virus [17]. A preliminary study showed that extract of

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a 50 mg/mL solution of *R. scutellariae* had strong trophozocida effects against *Acanthamoeba royreba* but was less effective against *A. castellani* and *A. polyphaga* (unpublished data presented at 15th APCCMI 2014). Further work on components of this herb has also indicated that five of the six active ingredients had significant effects on the viability of *Acanthamoeba* trophozoites. Components of *R. scutellariae* have also been shown to be useful in the management of glaucoma [18] and so its effect on ocular tissues needs to be determined.

C. phellodendri contains QPAs and limonoids [19]. The latter agents have been shown to inhibit HIV replication [20] but to have little antibacterial activity [21].

The preliminary study described above indicated that these agents may be useful in the prevention and treatment of *Acanthamoeba* infections, in particular, microbial keratitis. However, before further studies can proceed, it is important to determine possible cytotoxic effects on ocular tissues. Cytotoxicity may be determined by measuring effects on cell viability using trypan blue exclusion staining, spectrophotometric absorbance of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolism, and flow cytometry with Annexin V-FITC and 7-AAD staining. Trypan blue can indicate loss of cell viability as once membrane integrity is lost, this dye can enter into the cell, indicating cell damage. It has been incorporated into the Vi-Cell system (Beckman Coulter, Harbor Boulevard, Fullerton, CA) which uses a high resolution digital camera to determine viability of individual cells [22].

MTT assay uses the cell's ability to reduce this compound to a coloured product which may be detected spectrophotometrically. MTT cannot pass through the cell membrane so its metabolism begins when the cell takes up the MTT molecule and metabolizes it to formazan. As this metabolism is energy dependent, it can only occur in viable cells [23]. Staining with Annexin V-FITC and 7-AAD only occurs if the cell membrane is damaged. Annexin V binds to phosphatidylserine on the inner leaflet of the cell membrane which is projected externally during apoptosis [24]. 7-AAD stains DNA and the level of staining of the cell allows cell viability to be determined. As an intact membrane prevents entry of the dye into the cell, staining indicates membrane disruption. For 7-AAD to stain, DNA must be intact but with the cell membrane and nuclear envelope disrupted, signaling late stage of cell death. Staining of Annexin V only shows that the cells have entered apoptotic phase and there is no hydrolysis of DNA [25].

Immortalized human corneal epithelial cells (HCEC) allow for an extended lifespan of donated human epithelial cells from corneal cadaver donor tissue which overcomes the problem associated with primary cell culture [26]. They have been shown to have no difference in morphology or functions from primary cell cultures and so are widely used to investigate cytotoxic effects of drugs and other products.

In this study, all three methods of assessment of cell damage were employed to determine possible cytotoxic effects of the three TCM herbs.

2. Methods

2.1. Preparation of herbal solutions

The herbs were purchased as freeze dried granules made from the appropriate parts of the plant used in TCM. All preparations had been checked by DNA sequencing to ensure purity. Hot water extracts of the three herbs were prepared by suspending 10 g of the powdered herb in 100 mL of distilled water at 80 °C for 1 h. Extraction using hot water has been shown to be effective against bacteria and fungi to confirm the presence of active ingredients, mass spectrometry/gas chromatography was performed and the presence of six active agents was confirmed. Following extraction, the solutions were centrifuged at 3500 rpm/15 min at room temperature and the supernatant filtered and refrigerated at 4 °C for up to two days. As two of the major physiological stresses leading to cell damage are pH and osmolarity, it is important that they are optimized before attempts are made to assess cytotoxicity of a compound.

The solutions were adjusted with phosphate buffered saline (PBS) to balance the osmolarity to 300 mOsm/L, which is the osmolarity of normal tears, and provide testing concentrations of each herb of 8%, 5%, 3%, and 1%. The initial pH of the extracts ranged from 5.2–7.0, depending on the herb, but following adjustment with PBS the range was 6.5–7.1 and so no further adjustments were performed.

2.2. HCEC cultures

HCEC cultures (ATCC CRL 11,135) were prepared on cell culture plates pre-coated with 0.1 mg/L fibronectin, 0.1 mg/L bovine collagen type I and 0.1 mg/L bovine serum albumin. The cells were cultured in keratinocyte-serum free medium (Gibco 17,005-042; Life Technologies, Grand Island, NY, USA) supplemented with 0.5 mg/L bovine pituitary extract BPE, and 50 mg/L epidermal growth factor. Growth was enhanced by addition of 500 ng/mL of hydrocortisone and 0.05 mg/L of human insulin. Cells were grown at 37 °C with 5% CO_2.

2.3. Vi-Cell

HCEC were grown to a confluent monolayer in pre-coated 24-well plates, the culture medium removed and cells washed twice with PBS. Cells were then exposed herbal extract at concentrations of 3%, 5%, and 8% by adding 200 μL of the prepared herbal solutions to each well, each concentration being performed in duplicate. PBS was used as a control. The cells were incubated for 5 min at 37 °C in 5% CO_2. The testing solutions were removed and the cells washed twice with PBS, followed by addition of 200 μL of 0.05% trypsin/EDTA (Invitrogen, Thermo Fisher, Waltham, MA USA) to each well and incubation for 2 min at 37 °C with 5% CO_2. To terminate the effects of the enzyme, 300 μL of culture medium was added to each well. A 500 μL aliquot from each well was enumerated and viability assessed using the Vi-Cell.

2.4. MTT

Using a 96-well plate, each well was seeded with 10,000 HCEC in 200 μL of culture medium. The cells were allowed to settle for 48 h at 37 °C with 5% CO_2 before testing. The culture medium was removed and the cells washed twice with PBS. Each herbal solution was added to three wells with cells as triplicates and a further three wells without cells as colour controls. The concentrations used were 1%, 3%, and 5%, as 8% had been shown by Vi-Cell to cause interference with interpretation. PBS was used as a control. The plate was then incubated at 37 °C with 5% CO_2 for 5 min. The herbal extract was then removed and the well washed twice with PBS. To determine viability, 100 μL of the culture media containing 10% 12 mM MTT (Sigma–Aldrich, St Louis, MO, USA) was added to each well and to four empty wells as controls. The plate was incubated 37 °C with 5% CO_2 for 4 h. The medium was then removed and 100 μL of dimethylsulfoxide was added to each well to lyse the cells. After 120 s of shaking, the plate was scanned in a spectrophotometer at 570 nm and absorbance recorded.

2.5. Flow cytometry

HCEC were grown in a 24-well plate to confluence and then washed twice with PBS. Each herbal solution was added to two wells,