行政院國家科學委員會專題研究計畫 成果報告

Benzyl alcohol (triamcinolone 防腐劑) 導致視網膜色素上皮細胞毒性之分子機制和訊息傳遞之研究
研究成果報告(精簡版)

計畫類別：個別型
計畫編號：NSC 97-2314-B-006-035-
執行期間：97 年 08 月 01 日至 98 年 07 月 31 日
執行單位：國立成功大學醫學系眼科

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處理方式：本計畫可公開查詢

中華民國 98 年 10 月 23 日
行政院國家科學委員會補助專題研究計畫 成果報告

計畫名稱：Benzyl alcohol.（triamcinolone 防腐劑）毒性導致視網膜色素上皮細胞死亡之分子機制和訊息傳遞之研究

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成果報告類型（依經費核定清單規定繳交）：□精簡報告  □完整報告

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執行單位：成功大學醫學系眼科學科

中華民國98年07月31日
Purpose: To investigate the cytotoxicity and mechanisms of benzyl alcohol (BA; the preservative in commercial triamcinolone acetonide suspensions) on human retinal pigment epithelial (RPE) cells. Methods: Cultured human ARPE-19 cells were exposed to BA solutions, and the toxic effects were evaluated by trypan blue in situ staining and dye exclusion assays, TEM, acridine orange/ethidium bromide staining, DNA laddering, TUNEL staining, propidium iodide/annexin V staining and flow cytometry, caspase activity and inhibition assays, mitochondrial dehydrogenase and transmembrane potential assays, reactive oxygen species (ROS) assay, and Western blots. Results: RPE cell damage was dose- and time-dependent. BA 0.225 mg/mL, the clinically relevant concentration in TA following intravitreal injection, caused ultrastructural damage and impaired human RPE cell function at 2 hours; but BA 0.0225 mg/mL did not. BA 9.0 mg/mL, the concentration in commercial TA suspensions, was toxic within 5 minutes. The mechanisms of cell death were necrosis and apoptosis, mediated by ROS, mitochondria, caspase 8, and PARP. Conclusion: BA 0.225 to 9 mg/mL can damage RPE cells via necrosis and apoptosis. We suggest that, before a commercial TA solution is used intravitreally, the vehicle should be removed, particularly during macular hole surgery.
INTRODUCTION

Triamcinolone acetonide (TA) suspensions are widely used to treat a variety of vitreoretinal/macular diseases, including proliferative diabetic retinopathy, refractory diabetic or uveitic or pseudophakic macular edema, macular degeneration, retinal vein occlusion, and proliferative vitreoretinopathy. Instillation of crystalline TA particles also helps visualizing the vitreous, posterior hyaloid, epiretinal or internal limiting membrane during surgeries. Between 2004 and 2008, about 700 articles on such topics were added to the MEDLINE database.

The vehicle toxicity would be of concern for intravitreal TA administration, particularly for the retinal pigment epithelium (RPE) during macular hole surgery because the RPE layer directly contacts with vehicle-containing TA. We ever reported the cytotoxicity and efficacy of some ophthalmic agents. 1-10 In this study, we investigated the cytotoxicity and mechanisms of benzyl alcohol (BA), the preservative in commercial TA suspensions, on RPE cells.

MATERIALS AND METHODS

Human RPE cells (ARPE-19 cell line) were cultured, exposed to BA and examined for cytotoxic effects. The most frequently used TA suspensions such as Kenalog or Kenacort-A (40 mg/mL) contain BA 9 mg/mL. When 0.1 mL is injected into a 4-mL vitreous cavity, the intravitreal concentration of BA is 0.225 mg/mL. We furthermore tested its 1/10 dilution (0.0225 mg/mL).

For in vivo experiments, male young adult Sprague-Dawley rats were injected with 0.01 mL (equivalent to 0.1 mL for intravitreal injection in human eyes) of BSS or BA into the vitreous cavity. Two hours later, the eyeballs were harvested for H & E staining and TUNEL staining.

Question 1: Dose BA damage RPE cells?

Exp 1. Trypan blue in situ staining and dye exclusion assays

Question 2: Is the RPE damage caused by necrosis or apoptosis?

Exp 2. Acridine orange/ethidium bromide staining

Question 3: Does apoptosis participate in the RPE cell death?

Exp 3. TUNEL staining

Question 4: How much is necrosis or apoptosis involved in the RPE cell death?

Exp 4. Propidium iodide/annexin V-FITC staining and flow cytometry

Question 5: What kinds of caspases are involved in the apoptosis?

Exp 5. Caspase activation and inhibition assays

Question 6: What are the functional changes in organelles such as in mitochondria during cell death?

Exp 6. Mitochondrial dehydrogenase and transmembrane potential assays
Question 7: Do reactive oxygen species (ROS) play a role in the cell death? 
*Exp 7. ROS production by chemiluminescence analyzer system*

Question 8: Does *apoptosis-inducing factor* play a role in the cell death? 
*Exp 8. Apoptosis-inducing factor/DAPI staining*

Question 9: What kinds of common apoptotic molecules are involved in the signaling pathway? 
*Exp 9. Western blot analysis*

Question 10: What are the ultrastructural changes? 
*Exp 10. Transmission electron microscopy (TEM)*

Question 11: Can the cell death be observed in vivo? 
*Exp 11. Rat model*

**RESULTS**

1. **Trypan blue in situ staining and dye exclusion assays**
   
   On the trypan blue in situ staining (*Fig 1, A*), only a few cells exposed to BSS (control) stained with trypan blue, indicating cell death, and the dead cells were of the same size and shape as living cells. Results were similar for cultures exposed to BA 0.0225-3 mg/mL for various periods of time. However, exposure to BA 9 mg/mL resulted in more trypan blue-stained dead cells and more shrunken or even lysed cells, which increased over time.

   On the trypan blue dye exclusion assay (*Fig 1, B*), the proportions of stain-positive cells among those exposed to BA 0.0225-3 mg/mL were not statistically different from the control. However, for cells exposed to BA 9 mg/mL for 5 minutes or longer, the proportions of dead cells were significantly higher.

2. **Acridine orange/ethidium bromide staining**

   Almost all of the cells exposed to BSS remained viable (with a green nucleus and green cytoplasm), whereas exposure to increasing concentrations of BA resulted in increasing proportions of cells with orange-red fluorescence (*Fig 2*). Exposure time did not affect the degree of fluorescence in cells exposed to BA 0.0225-3 mg/mL, but it led to increasing fluorescence over time for cells exposed to BA 9 mg/mL. Nevertheless, these cells retained normal nuclear architecture without nuclear condensation or fragmentation, indicating cell death mainly by necrosis.

3. **TUNEL staining**

   On the TUNEL staining (*Fig 3*), many of the cells exposed to BA 0.225 mg/mL or high concentrations showed green fluorescent nuclei, indicating DNA fragmentation characteristic of late apoptosis. In addition, there was a dose dependency on the concentrations of BA. However, there was no difference between 2- and 6-hour exposures.
4. Propidium iodide/annexin V-FITC staining and flow cytometry

On the triple staining of propidium iodide, annexin V-FITC and Hoechst 33258, the cells exposed to BA 9 mg/mL showed green fluorescence, indicating early apoptosis (Fig 4, A). In addition, there was a time dependency between 2- and 6-hour exposures.

Propidium iodide/annexin V-FITC flow cytometry showed that, for the cells exposed to the culture medium (control), the mean percentages of single propidium iodide-positive, single annexin V-FITC-positive and double staining-positive cells were 1.7%, 0.5% and 1.2%, respectively (Fig 4, B). Exposure to lower concentrations of BA between 0.0225 and 3 mg/mL for 2 or 6 hours mildly increased these proportions (no more than 4.6%, 3.8% and 5.2%, respectively). However, BA 9 mg/mL significantly lead to 10.3% of single propidium iodide-positive cells after 2 hours of exposure, 19.0% of single annexin V-FITC-positive cells after 6 hours of exposure, and 64.2-71.4% of double staining-positive cells, indicating that BA induced not only necrosis but also apoptosis.

5. Caspase activation and inhibition assays

On the caspase activation assay, BA 9 mg/mL mostly increased the activity of caspase 8 after various time periods of exposure, particularly leading to 1.23 folds of induction after 30 minutes of exposure (Fig 5, A). In addition, the activities of caspases 5 and 9 increased at some of the time points.

On the caspase inhibition assay by flow cytometry for propidium iodide-stained cells (Fig 5, B), the percentages of sub-G0/G1 (M1 peak) apoptotic cells were 14.0% after exposure to BA 9 mg/mL for 2 hours, compared to 2.2% after exposure to the culture medium (control). Among the various caspase inhibitors, z-VAD-fmk (broad inhibitor) and z-IETD-fmk (caspase 8 inhibitor) could greatly reduce the percentages of apoptotic cells (2.3% and 3.3%, respectively), and z-YVAD-fmk (caspase 1 inhibitor), z-DEVD-fmk (caspase 3 inhibitor) and z-LEHD-fmk (caspase 9 inhibitor) could moderately inhibit apoptosis (5.3-8.0%). However, z-VDVAD-fmk (caspase 2 inhibitor) had no significant effects.

6. Mitochondrial dehydrogenase and transmembrane potential assays

On the MTT assay (a mitochondrial dehydrogenase assay), exposure to BA 0.225 mg/mL for 2 hours or 9 mg/mL for 5 minutes or longer caused lower optical densities (i.e., lower mitochondrial function, or higher cytotoxicity) compared to BSS (Fig 6, A).

On the mitochondrial transmembrane potential assay (Fig 6, B), exposure for 2 hours to BA 3 mg/mL and 9 mg/mL lead to 25.7% and 55.6% reduction, respectively, of the mitochondrial transmembrane potential at a dose-dependent manner, compared to 14.1% exposed to the culture medium (control). Pretreatments with z-IETD-fmk (caspase 3 inhibitor) and z-VAD-fmk (broad caspase inhibitor) were similarly effective to decrease the reduction of mitochondrial transmembrane potential.

As for the effects of mitochondrial stabilizers, cyclosporine A reduced BA-induced apoptosis at a dose-dependent manner on flow cytometry of propidium iodide-stained cells (Fig 5, B).

7. ROS assays
BA 9 mg/mL greatly increased the production of ROS in cultured RPE cells within 15 minutes, detected by the chemiluminescence analyzer system (Fig 7). However, this induction could be significantly reduced by the pretreatment with an ROS inhibitor, diphenyleneiodonium (DPI).

As for the inhibitory effects of DPI on the percentages of apoptotic cells on the propidium iodide-staining flow cytometry, it greatly decreased the amount of BA-induced apoptosis (1.8% versus 14.0% of apoptotic cells induced by BA 9 mg/mL for 2 hours; Fig 5, B). On the mitochondrial transmembrane potential assay, DPI could also significantly decrease the level of potential reduction (29.9% versus 55.6% induced by BA 9 mg/mL for 2 hours; Fig 6, B).

8. Apoptosis-inducing factor/DAPI staining

Of the cells exposed to either the culture medium (control) or BA 9 mg/mL for 2 or 6 hours, AIF remained distributed in the cytoplasm without translocation into the nuclei (Fig 8), indicating that AIF did not play a role in the cell death.

9. Western blot analysis

Western blots of the cultured cells exposed to BA 9 mg/mL showed an increased amount of activated caspase 8 after 5 minutes to 1 hour and poly (ADP-ribose) polymerase (PARP) after 5 minutes to 2 hours (Fig 9). On the contrary, the amount of caspase 3 proform was decreased after 5 minutes of exposure. In addition, Bcl-2 was also decreased after 1- or 2-hour exposure.

As for the amounts of AIF, P53, CD95 or Bax in the RPE cells, they remained unchanged.

10. TEM

TEM photomicrographs of cultured RPE cells exposed to BSS or BA solutions revealed that cells retained intact cell membranes, cytoplasm and nuclei after exposure to BSS or BA 0.0225 mg/mL, either for 5 minutes or 2 hours, or BA 0.225 mg/mL for 5 minutes (Fig 10). However, cells exposed to BA 0.225 mg/mL for 2 hours or 9 mg/mL for 5 minutes had swollen organelles in the cytoplasm, indicating early ultrastructural damage.

11. In vivo experiments

The H & E staining of the rat retina showed no significant light microscopic changes after 2 hours following intravitreal injection of BSS or BA 9 or 30 mg/mL (Fig 11). All of the structures in the neurosensory retina and RPE remained intact.

The TUNEL staining showed no finding after intravitreal injection of BSS. However, a number of TUNEL-positive green-fluorescent cells were noted at the outer nuclear layer (nuclei of photoreceptors) and RPE after 2 hours following intravitreal injection of BA 9 or 30 mg/mL (Fig 11). In addition, the apoptotic features were more evident in BA 30 mg/mL than in BA 9 mg/mL, indicating a dose-dependent manner.

DISCUSSION

This study showed that BA 0.225 mg/mL, the clinically relevant concentration for
intravitreal injection of TA, caused ultrastructural damage and impaired human RPE cell function within 2 h of exposure, whereas exposure to a 10-fold dilution (0.0225 mg/mL) did not. Exposure to BA 9.0 mg/mL (the concentration in commercial TA suspensions) resulted in toxic changes evident on each assay. Our in vitro and in vivo findings have significant implications for intravitreal use of TA suspensions.

**Experimental toxicity of BA for ocular tissues**

The commercial TA suspensions are initially developed for intra-articular or intramuscular injection, and their wide intraocular applications are still off-label use. We demonstrated that BA 9 mg/mL caused extensive lysis of rabbit corneal endothelial cells within 1 minute, and further found that preserved TA lead to RPE cytotoxicity which could be eliminated by removing the vehicle from TA suspensions. Others’ in vivo studies showed that BA produced electroretinographic and structural damages in the rabbit eyes.

**Mechanisms of cell death**

Based on our previous study, the major mechanism of RPE cell death induced by BA was necrosis, occurring within only 5 minutes. Furthermore, we herein show that a longer exposure to BA would also lead to another kind of cell death, i.e., apoptosis. In this study, several important mediators/molecules/organelles participating RPE cell death were ROS, caspases 8 and 3, mitochondria, and PARP. We propose the signaling pathways in Fig 12, in which ROS can lead to not only necrosis but also apoptosis. Then ROS can directly or indirectly activate caspase 8, which truncates Bid to impair the mitochondrial transmembrane potential and dehydrogenase functions. The mitochondrial damage can further release a more amount of ROS at a vicious cycle, leading to more severe cell damage. Furthermore, the mitochondria can lead to apoptosis through an AIF-independent pathway, perhaps involving some molecules such as Smac/DIABO or Omi/Htr, which needs further investigation. Alternatively, the mitochondria can release cytochrome c, which activates caspases 9 and 3 and PARP to result in apoptosis. Another possible pathway is that the caspase 8 can bypass the mitochondria and activates caspase 3 to induce apoptosis.

**Clinical evidence of BA toxicity for ocular tissues**

Intravitreal injection of BA-preserved TA can cause toxic endophthalmitis in 1.1%-6.7% of cases. TA vehicles contain 0.9% (9 mg/mL) BA as the preservative, 0.75% carboxymethylcellulose sodium and 0.04% polysorbate 80 as the suspending agents, and sodium hydroxide/hydrochloric acid to adjust pH. BA may be the most likely to induce toxic endophthalmitis. This suspicion was supported by two large-scale studies, in which there was no intraocular inflammation associated with 600 and 1135, respectively, intravitreal injections of TA suspensions from which the vehicle had been removed.

**Clinical relevance of findings**

Even for an intact neurosensory retina, small lipophilic molecules such as TA and BA can
easily diffuse through the whole retina and potentially damage the retinal neural cells and RPE cells. As for in the TA-assisting macular hole surgery, the localized concentration of BA directly contacting the naked RPE may be as high as 9 mg/mL, which can damage the RPE cells within minutes, as shown in this study. Therefore, we suggest removal of the vehicle before intraocular use of TA, either by standstill (sedimentation), by density-gradient centrifugation, or by filtration method. All of them can similarly remove 90% of BA in the TA vehicle. The remaining 10% (intravitreal concentration of 0.0225 mg/mL), which our results show, is non-toxic to RPE cells. Nevertheless, we suggest additional safety measures such as instilling autologous blood, serum or viscoelastic substance within the macular hole before TA-assisting internal limiting membrane peeling.

CONCLUSION

Our in vitro and in vivo studies showed that BA causes time- and dose-dependent damage to RPE cells by necrosis and apoptosis, and that even a clinically relevant low concentration of BA 0.225 mg/mL causes ultrastructural and functional damage to cultured human RPE cells within the first 2 hours of exposure. However, no such toxicity was seen with 2 hours of exposure to a 10-fold dilution (0.0225 mg/mL). We therefore recommend that only vehicle-removed TA preparations should be used in the eye.
REFERENCES
13. 博士論文 題目: 曲安奈德懸浮液及其防腐劑苯甲醇對眼部細胞之毒性 (Ocular toxicities of triamcinolone acetonide suspensions and their preservative benzyl alcohol)。作者：成功大學醫學院臨床醫學研究所博士班張義昇。指導教授：眼科曾順輝教授和生化所吳昭良教授。畢業日期：2008.05.24。得獎：成大醫學院博士班研究生論文競賽優勝獎 (Outstanding Research Award)
FIGURE LEGENDS

Fig 1. A, Trypan blue in situ staining of cultured human RPE cells after exposure to control (BSS) or BA. Damage is indicated by trypan blue stain in the nucleus and morphological changes. Arrows point to lysed cells. B, Trypan blue dye exclusion assay determining percentage of dead RPE cells. N= 5; * P < 0.05, Student's t-test versus control of the same exposure time.

Fig 2. Acridine orange/ethidium bromide staining of RPE cells after exposure to BSS or BA. Viable cells have an intact green nucleus and green cytoplasm. Punctate orange-red fluorescence in the cytoplasm indicates compromise of cell membrane integrity and accumulation of acridine orange within the lysosomes. Orange-red fluorescence completely filling the cytoplasm and nucleus indicates extensive cell damage by necrosis, although the orange-red nuclei have normal nuclear architectures without signs of apoptosis such as condensation or fragmentation.

Fig 3. TUNEL/DAPI staining of RPE cells. The cells with green-fluorescent nuclei are undergoing late apoptosis.

Fig 4. A, Propidium iodide/annexin V-FITC/Hoechst 33258 staining of RPE cells. The cells with green fluorescence are undergoing early apoptosis. Arrows indicate the non-apoptotic cells after exposure to BA 9 mg/mL. B, Flow cytometric analysis of RPE cells stained with propidium iodide and annexin V-FITC after exposure to the control solution or BA 0.0225-9 mg/mL. N= 5; * P < 0.05, Student's t-test versus control.

Fig 5. A, Caspase activation assay of RPE cells exposed to the control solution (culture medium) or BA 9 mg/mL for between 30 minutes and 6 hours. The Y axis represents the ratio of optical densities of BA/control, indicating the fold of caspase induction for each group. B, Caspase inhibition assay of RPE cells using propidium iodide staining and flow cytometric analysis, after exposure to the control solution or BA 9 mg/mL for 2 hours. The Y axis represents the percentages of sub-G0/G1 apoptotic cells. The inhibition groups of cells have been pretreated by caspase inhibitors, reactive oxygen species inhibitor (diphenyleneidonium, DPI), mitochondrial stabilizer (cyclosporine A, CSA), or GSK inhibitors (LiCl and BIO). N= 5.

Fig 6. A, MTT assay determining proportions of functional RPE cells. N= 5; * P < 0.05, Student's t-test versus control of the same exposure time. B, Mitochondrial transmembrane potential (MTP) assay labeled by rhodamine 123 on flow cytometry. N= 5; * P < 0.05, Student's t-test versus BA 9 mg/mL of the same exposure time.

Fig 7. Reactive oxygen species (ROS) production by chemiluminescence analyzer system. N= 5; * P < 0.05, Student's t-test. DPI= diphenyleneidonium, an ROS inhibitor.

Fig 8. Apoptosis-inducing factor/DAPI staining showing AIF distributed in the cytoplasm without translocation into the nuclei.

Fig 9. Western blots of RPE cells after exposure to the control or BA 9 mg/mL. Some common apoptotic markers are examined, including apoptosis-inducing factor (AIF), P53, CD95, Bax, Bcl-2, caspases 8 and 3, and PARP. Red marks indicate significant changes of the amounts of proteins.

Fig 10. TEM photomicrographs of RPE cells exposed to BSS or BA. Swollen organelles (white arrows) indicate damages to the permeability of cell membrane, and electron-dense granules (black arrow) indicate possible accumulation of BA within the cells.

Fig 11. Photomicrographs of the rat retina after intravitreal injection of BA 9 or 30 mg/mL for 2 hours. Left, H & E satin. Right, merge of TUNEL and DAPI stains. Arrows indicate
TUNEL-positive green-fluorescent cells at the outer nuclear layer (ONL) and RPE.

Fig 12. Proposed molecular mechanisms of BA-induced cell death and signaling pathways on human RPE cells.