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ENHANCED FLUORESCENCE IMAGING WITH DMSO-MEDIATED OPTICAL CLEARING

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Recent studies have demonstrated that topical application of glycerol on intact skin does not affect its optical scattering properties. Investigators from our research group recently revisited the use of dimethyl sulfoxide (DMSO) as an agent with optical clearing potential. We address the use of optical clearing to enhance quantitation of subsurface fluorescence emission. We employed both *in vitro* and *in vivo* model systems to study the effect of topical DMSO application on fluorescence emission. Our *in vitro* experiments performed on a tissue-simulating phantom suggest that DMSO-mediated optical clearing enables enhanced characterization of subsurface fluorophores. With topical DMSO application, a marked increase in fluorescence emission was observed. After 30 min, the fluorescence signal at the DMSO-treated site was 9× greater than the contralateral saline-treated site. This ratio increased to 13× at 105 min after agent application. In summary, DMSO is an effective optical clearing agent for improved fluorescence emission quantitation and warrants further study in preclinical *in vivo* studies. Based on outcomes from previous clinical studies on the toxicity profile of DMSO, we postulate that clinical application of DMSO as an optical clearing agent, can be performed safely, although further study is warranted.

Keywords: Glycerol; spectroscopy; fluorescein; toxicity.

1. Introduction

Hyperosmotic agents have been used to induce a reduction in the reduced scattering coefficient of biological tissue. Several published studies[1–12] have demonstrated that this optical clearing effect can increase the penetration depth and image contrast associated with several optical techniques.

Recently, Matsui *et al.*[13] studied the use of topically-applied chemical agents, specifically glycerol and a polypropylene glycol:polyethylene glycol (PPG:PEG) mixture, to improve near-infrared fluorescence image contrast. They reported that topical application of either agent did not result in any significant improvement in optical image contrast.

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Based on their negative findings, they concluded that optical clearing with such agents is ineffective for *in vivo* image-guided surgery.

For glycerol, the most widely studied optical clearing agent (OCA), the findings by Matsui *et al.*[13] actually are in agreement with those reported in previous studies[2,12,14,15]: topical application of glycerol on intact skin does *not* affect its optical scattering properties. Furthermore, the PPG:PEG OCA reported previously[16,17] as having an optical clearing effect with topical application, was an amphiphilic compound which resulted from covalent linkage of PPG and PEG molecules, and not a mixture of the two agents, as apparently used by Matsui *et al.*

Nevertheless, the negative outcome reported by Matsui *et al.*[13] underscores the primary limitation of *in vivo* optical clearing: agents with optical clearing potential (OCP) do not penetrate the intact stratum corneum of skin. Investigators from our research group recently revisited the use of dimethyl sulfoxide (DMSO) as an agent with OCP.[2] Use of DMSO was inspired by the surprising lack of proven toxic effects associated with the agent (see below). With topical DMSO application, our *in vitro* data demonstrated a marked reduction in the bulk reduced optical scattering coefficient (μ_s') of human skin samples. With use of an *in vivo* dorsal window chamber model, we demonstrated improved visualization of subsurface (∼0.5 mm deep) microvasculature with topical DMSO application.

In this report, we address the original problem studied by Matsui *et al.*[13]: the use of optical clearing to enhance quantitation of subsurface fluorescence emission. We employed both *in vitro* and *in vivo* model systems to study the effect of topical DMSO application on fluorescence emission. Based on results from our previous study,[2] we hypothesized that fluorescence emission application would increase, as compared to the negative control (saline) experimental condition.

2. Materials and Methods

2.1. *In vitro tissue phantom experiments*

For *in vitro* experiments, cryopreserved, dermatomed human skin (Science Care, Phoenix, AZ) was thawed to room temperature (∼26°C). With a single-edged razor blade, the skin was cut into approximately 2.5 cm × 2.5 cm samples. The thickness of each sample was measured by placing it between two glass slides, clamping the preparation with binder clips to provide consistent compression and uniform thickness, and measuring the preparation thickness with a micrometer (Mitutoyo, City of Industry, CA). To minimize systematic error and maximize repeatability of the measurement method, we recorded the preparation thickness after one click of the fine adjustment screw on the micrometer. By calculating the difference between the thickness of the preparation and the slides, we determined the thickness of each sample. The thickness ranged between 0.4 mm and 0.6 mm.

A silicone phantom was synthesized with dimensions of 9 cm × 9 cm × 1.5 cm in a Petri dish. A 1:10 ratio of P-4 Curing Agent/P-4 translucent silicone rubber (Eager Plastics, Chicago, IL) was used during phantom preparation. To achieve a reduced scattering coefficient of 4 mm^{-1}, silicon dioxide (SiO_2, TI602, Atlantic Equipment Engineers) was mixed by multiplying the total volume of the silicone rubber and curing agent by 0.002656 g/mL. Prior to phantom solidification, four plastic tubes (Tygon® formulation S-54HL), each with an inner diameter of 250 μm, were embedded within the phantom at depths of 1, 2, 3, and 5 mm. Each tube was offset in the lateral direction by 2 mm with respect to the immediately overlying tube.

As a representative fluorescent target, we used fluorescein (2.33 mg/mL). For each experiment, a freshly thawed skin sample was placed on top of the phantom to insure that the tubes were covered. To collect data, a multispectral imaging camera (Nuance, CRI, Woburn, MA) was used. A fiber-optic illuminator equipped with a blue (488 nm) excitation filter, served as an excitation source, and fluorescence emission images were collected with the camera liquid-crystal tunable filter set at 530 nm. The camera exposure time was fixed at 230 ms and the field of view remained constant at 5.5 cm × 5.5 cm.

The skin samples were treated either with 14 M DMSO (experimental condition) or isotonic saline (negative control). Agents were applied topically and held in place with circular glass chambers (1.5 cm in diameter) in the center of each skin sample. A total of six experiments were performed. Images of the samples were taken at 0, 15, 30, and 45 min after topical agent application, in addition to baseline images of the phantom alone to serve as measures of the "true" fluorescence. Prior to

agent application, we identified regions from which intensity line profiles were extracted. To account for potential nonuniformities in optical excitation, a flat-field correction was performed for each image. Due to the rigid positioning of both the camera and phantom, the same regions were analyzed in all subsequent images.

2.2. *In vivo fluorescence imaging experiments*

Adult (∼25–30 g) C3H mice were used. Each animal was anesthetized with isoflurane gas followed by an injection of a ketamine/xylazine (4:3 ratio, 0.4 mL/100 g mass) cocktail. After removing the fur from its dorsum, each animal was given a retro-orbital injection of ∼0.5% fluorescein isothiocyanate (FITC)-dextran, a common fluorophore used in optical microscopy. Prior experiments with installed window chambers demonstrate that this injection route is a reliable intravascular delivery method for FITC-dextran. The animal was placed on a custom stage with the dorsum facing upwards. Hill Top chambers, used in pharmacological studies as donor compartments,[2,18] were modified by removing a central 7 mm-diameter section, to facilitate image collection and reapplication of chemical agents to the site of interest. The modified chambers were placed over two contralateral sites on the mouse dorsum, as wells to hold either DMSO or saline. The chambers were constantly monitored for any leakage to insure consistent topical application.

3. Results and Discussion

Prior to agent application, fluorescence emission from the 1-mm-deep tube was barely distinguishable (Fig. 1), and emission from the 3- and 5-mm-deep tubes could not be detected. With saline application, the fluorescence signal did not change. In contrast, with DMSO application, a noticeable increase in fluorescence signal was observed from both the 1- and 2-mm-deep tubes. The largest increase in fluorescence signal occurred between the 15- and 30-min timepoints. A slight decrease in fluorescence is shown between the 30- and 45-min marks, but the signal remained well above the initial values. Fluorescence emission from the 3- and 5-mm-deep tubes remained obscured during the period of data collection. We anticipate that use of near-infrared fluorescence imaging may enable detection of these deeper tubes.

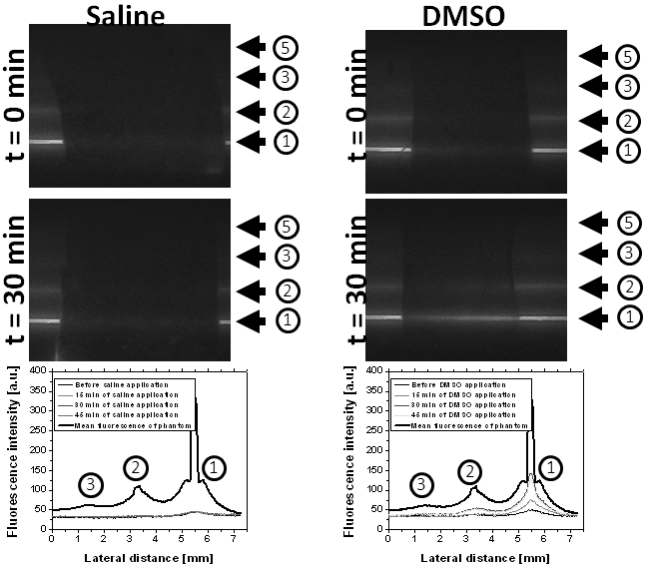

Fig. 1. DMSO-mediated optical clearing enables enhanced characterization of subsurface fluorophores. Isotonic saline (left) and 14 M DMSO (right) was applied topically to *in vitro* human skin samples placed on top of a silicone phantom with embedded tubes filled with fluorescein. The change in fluorescence was observed over a 45-min application period. We reduced the imaging data by taking averaged line profiles within the same region of interest in each image. The "mean fluorescence of phantom" line profile data were collected directly from the phantom itself (i.e., without overlying skin); these data demonstrate that the fluorescence emission from the fluorescein was stable during the imaging period. The mean coefficient of variation of the phantom fluorescence signal was less than 2.5%.

With the same excitation and imaging setup described above, *in vivo* fluorescence images were taken every 15 min, over a 120-min period. Imaging and agent application started 10 min after FITC-dextran administration. Isotonic saline was applied in one Hill Top chamber and 14 M DMSO in the contralateral chamber. Prior to image collection, the application sites were wiped clean with Kimwipes; each agent was reapplied immediately afterwards.

With topical DMSO application, a marked increase in fluorescence emission was observed (Fig. 2). In contrast, at the contralateral saline site, fluorescence slightly increased at some pixels, but the overall mean signal slightly decreased with time. After 30 min, the fluorescence signal at the DMSO-treated site was 9× greater than the contralateral saline-treated site. This ratio increased to 13× at 105 min after agent application.

In these experiments, we routinely observed focal regions of increased fluorescence emission at

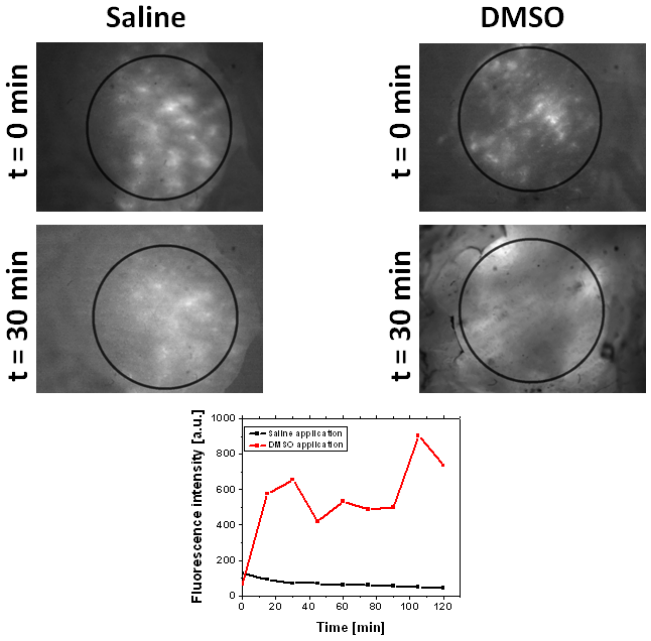

Fig. 2. DMSO-mediated optical clearing increases *in vivo* fluorescence emission of intravascular FITC-dextran. (Top) Fluorescence emission images collected prior to and 30 min after topical administration of either isotonic saline or 14 M DMSO on intact mouse dorsal skin. Field of view: 1.5 cm × 1.5 cm. (Bottom) Quantification of fluorescence intensity on mouse dorsum after treatment with either isotonic saline or DMSO, over a 120-min duration. Fluorescence intensity values taken from the region enclosed in the black-outlined circles in each image were averaged. The line between each data point is used to guide the reader's eye and is not indicative of actual fluorescence intensity values between each point.

the DMSO-treated site (Fig. 2). These regions may correspond to the location of vasculature, hence resulting in improved resolution of microvasculature in a similar fashion to our previous experiments.[2] Also, as opposed to the monotonic decrease in fluorescence signal at the saline-treated site, we observed instead a highly-dynamic signal (Fig. 2, bottom graph). The source of these fluctuations is currently unknown. Future experiments are warranted to study these *in vivo* phenomena in more detail.

A common assumption about DMSO is that its toxicity profile is unacceptable for clinical use. In fact, information on the material safety data sheet (MSDS) on DMSO,[19] suggests that DMSO actually has low toxicity and is only a mild skin irritant. DMSO is FDA-approved for treatment of interstitial cystitis,[20] at a concentration of 50% w/w (Rimso-50, Bioniche Pharma USA LLC, Lake Forest, IL). In this treatment, 50 mL of the 50% DMSO solution is instilled directly into the bladder, suggesting that human biological tissue can safely tolerate both large quantities and relatively high concentrations of DMSO.

DMSO is also routinely used as a cryoprotectant during preservation of various cell types, including blood stem cells[21] and bone marrow mesenchymal stem cells.[22] DMSO is currently used as a vehicle for transdermal delivery of drugs in novel therapeutic protocols, such as photoactivated agents.[23]

Based on the peer-reviewed literature, the degree of DMSO toxicity is less clear, as a wide range of toxicity profiles have been reported. Several groups report that high concentrations of DMSO are well tolerated by humans. Ludwig *et al.*[24] applied a bandage with 90% DMSO to eight subjects and observed mild skin irritation as the only complication. Bertelli *et al.*[25] studied 144 patients who were treated with a seven-day regimen involving 99% DMSO, designed to prevent further extravasation of chemotherapeutic agents. The only side effect involved mild skin irritation and the characteristic garlic odor which occurs with DMSO absorption by the body. Furthermore, Karande *et al.*[26] evaluated 102 potential chemical penetration enhancers for transdermal delivery, including DMSO, and found that the irritation potential of DMSO was on par with oleic and linoleic acids, which are common.

On the other hand, several groups provide reasons to caution against clinical use of DMSO. With intravenous administration of DMSO, blood serum changes were observed in one subject.[27] Jacoby and Weiss[28] provide preclinical data which suggest that DMSO can either promote or inhibit tumor formation, depending on the manner in which DMSO is utilized. Serbye *et al.*[29] demonstrated that DMSO can irritate gastric tissue when applied directly, even at concentrations as low as 5%.

Based on the collective data contained in this report and in the published literature,[2,9] it is evident that application of DMSO is an effective optical clearing method to enhance our ability to both visualize and quantify subsurface features. The collective data further demonstrate that this enhancement can be achieved with one-time application of DMSO over short (<60 min) exposure times. For preclinical studies, we propose that DMSO-mediated optical clearing can be used to enable collection of weak optical signals (i.e., bioluminescence, fluorescence) which may otherwise be

undetected with conventional imaging or spectroscopic measurements.

Hence, based on outcomes from previous clinical studies investigating the toxicity profile of DMSO,[24,25] we postulate that clinical application of DMSO as an optical clearing agent, can be performed safely. To minimize potential complications, we propose that DMSO should be applied infrequently (i.e., once every four to six weeks) and over short exposure times, and that a medical-grade formulation (i.e., 99.9% purity) be used and carefully maintained, to minimize the occurrence of potential complications due to the fact that DMSO is an excellent solvent.

In summary, DMSO is an effective optical clearing agent for improved fluorescence emission quantitation. Our *in vitro* data (Fig. 1) suggest that this fluorescence enhancement can occur for fluorophores at depths of up to 2 mm, although it is unclear as to how well these data translate to *in vivo* use. Our *in vivo* data collected from intact mouse skin, demonstrate that DMSO-mediated optical clearing can enhance substantially fluorescence emission in a realistic animal model, suggesting the potential efficacy of DMSO as an imaging adjuvant in preclinical and clinical studies. Future efficacy and safety experiments are warranted.

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