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Molecular iodine is not responsible for cytotoxicity in iodophors

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SUMMARY

Background: Ten percent povidone-iodine (PVP-I) was initially promoted as ‘tamed iodine’ as the chemical activity of the active biocide, uncomplexed or free molecular iodine (I₂), is reduced 30- to 50-fold compared with Lugol’s solution. The idea that I₂ is responsible for topical iodine staining and irritation remains widely held. However, there are no controlled studies that characterize the cytotoxicity and staining of the hydrophobic I₂ species compared with the other hydrophilic iodine species that comprise over 99.9% of the total iodine in topical iodine disinfectants.

Aims: To compare the staining properties of the I₂ species with other topical iodine disinfectants; to evaluate if the concentrations of I₂ in diluted PVP-I used to reduce severe acute respiratory syndrome coronavirus-2 in the nasal cavity are potentially cytotoxic; and to determine if high concentrations of I₂ can be delivered beyond the stratum corneum into the hypodermis, which could provide a mechanistic rationale for I₂ out-gassing.

Methods: Five liquid compositions that contained complexed and uncomplexed (free) I₂ in aqueous and non-aqueous carriers were used to evaluate the interaction of I₂ with mammalian cells in culture as well as human and pig skin.

Findings: Concentrations of I₂ (7800 ppm) that are 1500 times higher than that found in PVP-I can be applied to skin without irritation and staining. I₂ is not cytotoxic at concentrations >100 times higher than that found in PVP-I, and does not contribute materially to staining of skin at concentrations found in Lugol’s solution (approximately 170 ppm). I₂ can partition into hypodermis tissue, remain there for hours and out-gas from skin. PVP-I and Lugol’s solution are highly effective topical disinfectants, but do not facilitate diffusion of I₂ through the stratum corneum.

Conclusion: The maximum concentration of I₂ found in diluted PVP, approximately 25 ppm, is not cytotoxic or irritating. The potential clinical utility of I₂ has been limited by incorporating this broad-spectrum biocide into acidic aqueous formulations that contain numerous chemical species that contribute toxicity but not biocidal activity. I₂ can be delivered topically into hypodermis tissue without irritation.

Introduction

The global pandemic has induced clinicians to identify agents that can be used in the oral and nasal cavities [1–6].
Use of an iodine disinfectant is logical as: (i) iodine has established antiviral and antimicrobial activity; (ii) there is no evidence that bacteria can develop resistance to I$_2$ [7,8] as it reacts with several functional groups, thus providing simultaneous action against multiple molecular targets; (iii) iodine is a naturally occurring biomolecule synthesized in the follicular lumen and other iodine-concentrating tissues; and (d) iodine has a 200-year history of use in and on humans.

The nose is the primary nidus of infection for severe acute respiratory syndrome coronavirus-2, and while masks mitigate aerosol spread, they do nothing to reduce viral load in the nasal cavity [9,10]. The 3M Skin and Nasal Antiseptic is 5000 ppm available iodine iodophor that is painted on to the nasal cavity prior to surgery [11], and many clinicians have advocated for use of a diluted iodophor solution with 0.25–1.5% thiosulphate titratable as a prophylactic nasal lavage [12–16] for healthcare personnel. The US Food and Drug Administration has reviewed at least 27 clinical trials that use diluted povidone-iodine (PVP-I) [17] to control coronavirus disease 2019. Oral and nasal application of diluted PVP-I nasal spray and irrigation [18,19] has been adopted, and products with lower total iodine concentrations are also offered for this purpose (ioShield Nasal Antiseptic, Miami, FL, USA).

The thiosulphate titratable iodine in PVP-I can vary from the amount stated on the label by 35% as the US Pharmacopeia (USP) definition requires not less than 85% and not more than 120% of the labelled amount of iodine [20]. The term ‘iodine’ in this sentence means thiosulphate titratable iodine. However, triiodide, hypooiodous acid and I$_2$ are all reduced by thiosulphate, and the concentration of active biocide in PVP-I is 0.02–0.06% of the label claim. In addition, there is up to 6.6% iodide ion in PVP-I. Consequently, clinicians do not know the concentration of active biocide in the iodophor they use clinically. Nuckolls has highlighted the risks and limitations inherent in the simple dilution of PVP-I to generate a ‘low concentration’ iodine disinfectant which can have a 10-fold higher concentration of active biocide (i.e. I$_2$) [21].

Gottardi made the surprising discovery that topically applied I$_2$ can diffuse into skin and provide prolonged (>12 h duration) epidermal antibacterial activity due to a continuous flux or ‘back diffusion’ of absorbed I$_2$ from skin [22]. Dilution of an iodophor such as PVP-I results in an increase of I$_2$ concentration [23–25] that also increases absorption of I$_2$ into skin, as the I$_2$ flux is proportional to exposure time and concentration. The interaction of PVP-I with skin and wounds has been studied extensively, but these studies do not provide insight into the interaction of the I$_2$ species with skin. Only a limited number of studies have examined the topical properties of pure I$_2$. Duan et al. published acute dermal toxicity results for a low concentration (35 ppm) I$_2$ composition [26], and Uchiyama et al. examined the efficacy of a 400 ppm I$_2$ composition to reduce meticillin-resistant Staphylococcus aureus in the nasal carriage of mice [27]. The present study compares the interaction of I$_2$ with skin using (i) I$_2$-glycerine compositions and (ii) several commercially available aqueous iodine compositions.

**Methods**

**Staining of human skin by iodine compositions**

The forearm of a volunteer was cleaned with a Dacron wipe (75% alcohol) prior to application of the iodine compositions. Four glass vials (VWR Cat#66022-300) were held simultaneously against a human forearm for 3 min. Each vial contained 2 mL of the following compositions: (i) 10% PVP-I, (ii) 300 ppm I$_2$ in water, (iii) 7800 ppm I$_2$-glycerine, (iv) Lugol’s solution (3.3% I$_2$–6.6% NaI), and (v) USP tincture of iodine (47% ethanol; 2% I$_2$–2.4% NaI). After removal of the vials, the treated area of skin was cleaned twice with an alcohol Dacron wipe to remove residual material, and a stopwatch was started.

**Staining of pig skin with 66,000 ppm I$_2$-glycerine**

Thirty microlitres of a 66,000 ppm I$_2$-glycerine solution was applied to the epidermis of a 1.77-cm$^2$ piece of pig skin and spread over the surface for 20 s using a sterile disposable plastic loop. The material was retained on the skin surface for 3 min. The stratum corneum was removed, and a SenSafe Iodine Check test strip (Part No. 480018, Industrial Test Systems, Rock Hill, SC, USA) was contacted with the area of hypodermis tissue that had a purplish hue for 1 min to test for the presence of I$_2$.

**Staining of hypodermis pig skin tissue with I$_2$ compositions**

The epidermis and dermal layers of pig skin were separated and discarded. Cubes of hypodermis tissue 5 mm in width and length were sectioned, hydrated for 30 min in water, and patted dry. Five cubes of hypodermis tissue were submerged into 1 mL of each of the following three compositions: PVP-I, Lugol’s solution, and glycerine with 15,200 ppm I$_2$. Each piece of hypodermis tissue was removed after 15 min and rinsed thoroughly with water until a colour change was not detected. The tissue was contacted with a SenSafe Iodine Check test strip for 5 min to check for I$_2$.

**I$_2$ out-gassing from hypodermis tissue**

Ten cubes of hypodermis tissue 5 mm in width and length were hydrated for 30 min, patted dry, submerged in 2 mL of 15,200 ppm I$_2$-glycerine for 15 min, and then rinsed thoroughly seven times with water. At 15, 30, 45, 60, 75 and 90 min, and every 30 min thereafter until 210 min, a cube was removed and contacted with a SenSafe Iodine Check test strip for 5 min to test for the presence of I$_2$.

**Out-gassing of I$_2$ from pig skin**

Circular pieces (1.77 cm$^2$) of pig skin were cut, hydrated for 30 min at room temperature (20°C), and patted dry with a tissue. The pig skin was treated with 10 μL of a 66,000 ppm I$_2$-glycerine composition, spread over the epidermis for 20 s using a disposable plastic cell spreader. After 10 min, residual material was removed from the epidermis by wiping the top
surface of the pig skin with an alcohol Dacron wipe. Controls were treated with 10 μL of glycerine. Pig skin tissues were loaded individually into a vertical diffusion cell (VDC; Copley Transdermal Tester HDT 1000; Cat. No. 7290, Serial #50290) in an open configuration. Tissues were kept at a constant 34 °C in the VDC to mimic the temperature of human skin. Tissues were placed between two support washers, and the donor chamber was screwed into place. The occlusion disc was used intermittently by resting on top of the donor chamber to avoid loss of I₂ when not in use. I₂ diffusion from skin was measured using 1.0 mM N,N-dimethyl-p-phenylenediamine dihydrochloride (DPD) prepared daily. DPD reagent (1 mL) was loaded on to the pig skin in the donor chamber of the VDC to directly capture I₂ out-gassing from the epidermis. The DPD reagent was removed after a defined contact time that ranged from 30 s to 15 min, transferred to a cuvette, and absorbance was determined at 550 nm (Persee Analytics, Inc., Auburn, CA, USA; Model T6V). Pig skin was rinsed with two 1-mL DI-water rinses between DPD sampling to avoid cross-contamination. The I₂ flux (in μg/cm²-min) was determined by comparing the absorbance of unknowns with a standard curve prepared daily. The between-day mean (N=25) and standard deviation of the slope and intercept for the standard curve were 0.05217 A/μg ± 0.00236 and 0.01163 A ± 0.00959, respectively.

**Cytotoxicity of 24-h exposure to 1500 ppm I₂**

Three samples of 1500 ppm I₂-glycerine were evaluated for cytotoxicity using a direct contact test according to ISO 10993-5:2009 [28]. Sterile filter paper with a flat surface with a total surface area of 1.0 cm² was saturated with 0.1 mL of the test article, and placed directly on a cell culture monolayer of mouse fibroblasts (ATCC CRL-2648) in the centre of a 10-cm² well. Triplicate-positive (Encore Latex glove; Ansell Healthcare Products, LLC, Iselin, NJ, USA) and -negative (high-density polyethylene; USP, Lot KOM357) controls were tested in the same manner as the test articles. All wells were incubated for not less than 24 h at 37 ± 1 °C in a humidified incubator with 5 ± 1% CO₂. After incubation, the test articles and controls were removed from the wells, and the cell cultures were examined under an inverted microscope with 100X magnification for cytotoxic response.
Out-gassing of I$_2$ from human skin

The forearm of a volunteer was cleaned with a Dacron wipe (75% alcohol) prior to application of Lugol’s solution or PVP-I. A borosilicate glass vial with an inner diameter of 1.1 cm was contacted with an area on the forearm for 3 min, and the treated surface was cleaned with an alcohol Dacron wipe. An identical vial that contained 1 mL of DPD reagent was placed on the treated area for 3 min to capture I$_2$ diffusing from the skin. I$_2$ concentrations were determined as described for out-gassing of I$_2$ from pig skin.

Results

A glycerine composition with 7800 ppm of I$_2$ did not stain the skin on the forearm of a 70-year-old white male (Figure 1), in contrast to four other topical iodine formulations that contained much lower I$_2$ concentrations. PVP-I, which contains 2 ppm I$_2$, discoloured skin slightly more than a freshly prepared aqueous 300 ppm I$_2$ solution. Lugol’s solution, which contains 170 ppm free I$_2$, stained skin more deeply than any of the other materials, although it contains less (~56%) I$_2$ than the aqueous I$_2$ solution and 98% less I$_2$ than the glycerine composition. Lugol’s solution...
was detected for up to 2 h (Figure 4). The I$_2$ flux decreased of I$_2$ in a non-polar environment. Contacting a SenSafe Iodine sections of hypodermis tissue are consistent with the presence tissue that exhibited a purplish colour (Figure 2b). The purplish corneum, and mottled areas were observed in hypodermis deep reddish-brown stain (Figure 2a) formed on the stratum brown solution in water, in contrast to the violet colour discolouration from this higher level of I$_2$ dissipated substantially 10 min post application.

The I$_2$ flux from human skin measured after a 3-min 10% PVP-I exponentially with time, as reported previously by Gottardi [2].

Contacting thoroughly washed hypodermis tissue with a SenSafe Iodine Check test strip for 5 min yielded a

| Time post | I$_2$ ppm | Time to final |
| exposure (min) | score$^a$ | colour (s)$^b$ |
| 15 | 5 | 25 |
| 30 | 5 | 20 |
| 45 | 5 | 18 |
| 60 | 2 | 120 |
| 75 | 1 | 180 |
| 90 | 0.5 | 240 |
| 120–210$^c$ | 0 | 300 |

$^a$ SenSafe Iodine Check test strip incorporates a qualitative scale from 0 to 5 ppm I$_2$
$^b$ All observations monitored for 300 s.
$^c$ The 120-, 150-, 180- and 210-min time points yielded identical results.

and iodine tincture imparted the deepest stains; this implicates triiodide as the staining agent, as both of these products contain high concentrations of uncomplexed triiodide. Staining with I$_2$-glycerine required 15,000 ppm I$_2$ (data not shown), and the discoloration from this higher level of I$_2$ dissipated substantially 10 min post application.

Sodium thiosulphate has been used to remediate skin staining by topical iodine, which suggests that either triiodide, I$_2$ or both iodine species stain skin. I$_2$ presents as a yellowish-brown solution in water, in contrast to the violet colour observed in aprotic (non-polar) solvents or in the vapour phase. This study confirmed that pure I$_2$ can stain skin by applying 30 $\mu$L of a 66,000 ppm I$_2$-glycerine composition on pig skin. A deep reddish-brown stain (Figure 2a) formed on the stratum corneum, and mottled areas were observed in hypodermis tissue that exhibited a purplish colour (Figure 2b). The purplish sections of hypodermis tissue are consistent with the presence of I$_2$ in a non-polar environment. Contacting a SenSafe Iodine Check test strip with the purplish areas of hypodermis indicated the presence of I$_2$. The ability of I$_2$ to partition in hypodermis tissue was further tested by submerging cubes of hypodermis tissue from pig skin in PVP-I, Lugol’s solution and 15,200 ppm I$_2$-glycerine for 15 min. PVP-I did not stain hypodermis tissue, and a 5-min contact with a SenSafe Iodine Check test strip did not detect I$_2$ (Figure 3a). In contrast, staining was observed in hypodermis tissue treated with Lugol’s solution and 15,200 ppm I$_2$-glycerine (Figure 3b,c); a much darker stain was observed with the higher concentration of I$_2$ in the glycerine composition. Contacting thoroughly washed hypodermis tissue previously exposed to Lugol’s solution or 15,200 ppm I$_2$-glycerine with a SenSafe Iodine Check test strip for 5 min yielded a concentration of 1 and 5 ppm I$_2$, respectively; this is consistent with the idea that I$_2$ diffusion into hypodermis tissue is concentration dependent. After 15 min of exposure to 15,200 ppm I$_2$-glycerine, I$_2$ out-gassing was detected from cubes of hypodermis tissue (Table I) for up to 90 min.

I$_2$ out-gassing from pig skin treated with 66,000 ppm I$_2$-glycerine and maintained at 34 °C in a vertical diffusion cell was detected for up to 2 h (Figure 4). The I$_2$ flux decreased exponentially with time, as reported previously by Gottardi [2]. The I$_2$ flux from human skin measured after a 3-min 10% PVP-I application was 0.49 ± 0.047 $\mu$g/cm$^2$-min (N=5) at 0.5 min post application. Identical measurements made on human skin with Lugol’s solution at 0.5, 30 and 80 min post application were 5.39 ± 1.48, 1.32 ± 0.44 and 0.41 ± 0.24 $\mu$g/cm$^2$-min, respectively. These results are consistent with data reported previously by Gottardi [22], who demonstrated residual antibacterial activity against bacteria in water.

A 1500 ppm I$_2$-glycerine composition did not exhibit cytotoxicity in a 24-h direct contact test with mouse fibroblasts, which contrasts with cell-based cytotoxicity reports using commercially available topical iodine products [29].

Discussion

In a series of studies funded by the National Aeronautics and Space Administration, Thrall et al. demonstrated dramatic differences in the pharmacological and toxicological properties of orally administered iodide vs I$_2$ in the rat [30–32]; the results support the principal that the functional properties of an iodine composition depend upon the relative concentration of the different iodine species contained therein. Aceves and others have demonstrated dramatic differences in the behaviour of I$_2$ compared with iodide in different cell lines and tissues [30,31,33,34]. There are more than seven different iodine species in aqueous topical iodine disinfectants [35], in addition to other ingredients (e.g. surfactants, water, pH modifiers, etc.). All of the chemicals in these iodine products can contribute to their functional behaviour [36,37]. The present study observed the interaction of skin with pure I$_2$, and noted differences between the behaviour of pure I$_2$ and complex mixtures of iodine species (e.g. PVP-I).

Thiosulphate has been used successfully to treat iodine burns, which suggests that either triiodide, I$_2$ or both species are implicated as the causal skin staining agent as thiosulphate reduces both iodine species. The differences in human skin staining exhibited by the iodine compositions in this study are inconsistent with the long-held assumption that topical staining is due to I$_2$ [38]. The complete lack of staining from a 7800-ppm composition of I$_2$-glycerine contrasts dramatically with the deep persistent stain imparted by both Lugol’s solution and iodine tincture. This lack of staining at 7800 ppm I$_2$ combined with an absence of cytotoxicity with 1500 ppm I$_2$ in a 24-h direct contact test with fibroblasts suggests that high concentrations of I$_2$ can be used safely on skin if properly formulated. Skin staining from Lugol’s solution and iodine tincture may be due to the binding of triiodide to glycogen in the epidermis [39].

PVP-I is visible on skin but it does not stain skin, as diffusion of triiodide or I$_2$ into the epidermis is limited due to binding of I$_3$ by PVP. I$_2$ stains skin at higher concentrations but the resulting colour differs from that observed with triiodide staining; a difference in colour is also observed in in-vitro experiments that characterize glycogen-iodine complexes [40]. Systemic iodine exposure from topical iodine disinfectants such as PVP-I is well known [41–44]. The total iodine applied topically in a glycine-I$_2$ composition would be lower than with a PVP-I composition. For instance, a 1000 ppm I$_2$-glycerine solution would expose 100 $\mu$g of I$_2$ to a human hand based on 0.1 mL application on to a 400-cm$^2$ hand, which compares with approximately 10,000 $\mu$g of total iodine from PVP-I for an equivalent volume. However, a much higher percentage of the iodine in I$_2$-glycerine may be absorbed compared with PVP-I. Safran and Braverman evaluated thyroid
status during daily vaginal douching with PVP-I (0.3% thiosulphate titratable iodine) in 12 euthyroid volunteers for 14 days, and concluded that 5% of the applied iodine was absorbed [45]. A topical administration of 0.2 mL of an I2-glycerine composition containing 1000 ppm I2 used for hand disinfection would administer 200 µg of I2 on to skin. If one assumes 20% absorption as opposed to the 5% measured by Safran and Braverman, 40 µg would cross the stratum corneum. A maximum of 50% of this I2 could be available to partition into the thyroid [30–32], which equates to a total thyroid exposure of 20 µg of iodide which is approximately 15% of the recommended daily allowance. Consequently, it would be important to measure the potential for iodine exposure from repeat applications of a high I2-glycerine composition.

Gottardi first observed out-gassing of I2 from skin after application of Lugol’s solution [2]. In the present study, measurements of I2 flux from human skin treated with Lugol’s solution are consistent with Gottardi’s data. The purplish areas of coloured hypodermis in pig skin treated with 66,000 ppm I2-glycerine suggest the presence of I2, which is consistent with test results from the DPD test strip. The apparent stability of I2 absorbed into skin is ostensibly a surprising outcome as I2 is highly reactive with biological tissue. However, partitioning of I2 into regions of unsaturated lipid in the hypodermis could provide stability to I2, which partitions readily into oil and fat. It was observed that I2 partitioned into hypodermis tissue in proportion to the dose, as tissue treated with 15,200 ppm I2 contained more I2 than tissue treated with Lugol’s solution (170 ppm). The duration of out-gassing from pig skin treated with 66,000 ppm I2 was detected for approximately 3.3 h, compared with 4 h for hypodermis tissue treated directly with 15,200 ppm I2.

Gottardi mentioned that absorption and subsequent post-application diffusion of a topically applied antimicrobial over time is unique in the field of skin disinfection. Some researchers have reported a persistent antimicrobial activity on skin after use of iodine-based handwashes [46–49]. Converting hypodermis tissue into a material that releases I2 over time confers potential benefits for topical disinfection, and also offers the potential to: (i) treat infectious diseases, (ii) act as an anti-inflammatory as I2 can interrupt ligand signalling via iodination, and (iii) stimulate wound healing via the sustained delivery of free iodine at concentrations that retain antimicrobial activity without cytotoxicity [50].

It is known that the concentration of I2 increases as PVP-I is diluted up to approximately 1/100 [23]. Diluted PVP-I has been shown to be less cytotoxic than neat PVP-I; therefore, the literature provides indirect evidence that I2 is not the cytotoxic species in PVP-I. Additionally, cadexomer iodine, which contains a high concentration of I2 compared with 10% PVP-I, does not affect fibroblast viability and collage synthesis. In fact, cadexomer iodine stimulated secretion of proinflammatory cytokines (tumour necrosis factor-alpha) in human macrophages [23].

The characterization of PVP-I on product labels is not sufficient to ensure consistent results in cell-based cytotoxicity studies due to variations in pH (can vary between 1.5 and 6.5), iodide concentration, ionic strength, buffering capacity and I2 concentration [24,25]. Additionally, there is a 15% variance allowed in the level of titratable iodine under the definition in the USP. There is a legitimate concern that formulation variations among different PVP-I compositions (manufacturer-to-manufacturer and lot-to-lot) have influenced cytotoxicity study outcomes unbeknownst to researchers conducting these studies [29,51,52]. Equally problematic are studies that dilute a topical iodine composition and draw incorrect conclusions based on the assumption that I2 is stable for days after dilution [53]. Some manuscripts have thoughtfully explored the composition of PVP-I [54], but an insufficient understanding of the fundamental chemical properties of PVP-I almost certainly contributes to unfortunate clinical outcomes [55–57].
If one views I$_2$ as just another antimicrobial chemical, the lack of cytotoxicity observed for the 1500-ppm I$_2$-glycerine material may appear paradoxical given the elevated antimicrobial activity associated with such a high level of I$_2$. However, molecular iodine is a naturally occurring biochemical that can function as an antioxidant [58–60], an anti-inflammatory agent [61,62], an antiproliferative agent [35,63–68] and a differentiation agent [69–73]. There are more than 12 different tissues in humans with the ability to concentrate iodine. Based on a recommended daily allowance of 125 µg/day for iodide, the follicular lumen oxidizes 6.25 µg iodide/h into I$_2$ in a volume of approximately 7.5 mL, which equates to generating 900 ppm I$_2$/h which is approximately 2 orders of magnitude higher than the I$_2$ concentration found in PVP-I. Molecular iodine is the only widely used disinfectant molecule that plays an essential role in mammalian biochemistry [74] and, if formulated properly, can be used clinically at highly elevated concentrations.

In conclusion, the highest concentration of I$_2$ achievable from diluted PVP-I is approximately 25 ppm, which suggests that there is at least a 60-fold margin of safety for I$_2$ with respect to topical toxicity on human mucous membranes of the nasal cavity, as a 1500-ppm I$_2$ composition was not found to be cytotoxic. The staining and irritation associated with topical iodine disinfectants is not due to the I$_2$ species. There are potential clinical benefits that may accrue from the use of high concentrations of topically applied I$_2$.

Acknowledgements

The authors acknowledge the many original observations of Professor Waldemar Gottardi in the field of aqueous iodine chemistry, without which the observations in this study would not have occurred.

Conflict of interest statement

None declared.

Funding sources

This work was supported by I2Pure Corporation, Ashburn, VA.

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