Investigations into the effect of carbon monoxide exposure on bloodstain color: Implications for the Shroud of Turin

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Abstract

Hemoglobin (Hb) is the major protein present in red blood cells whose function is to transport oxygen throughout the body. The oxygenated form of hemoglobin is responsible for the typical red color of fresh blood that is normally observed. Carbon monoxide (CO) binds to hemoglobin with an affinity 250-300 times greater than that of oxygen, making it a highly competitive, toxic substance which can be lethal at even relatively limited levels of exposure. Blood drawn from patients experiencing CO poisoning exhibits a "cherry red" appearance, which is often useful in the initial diagnosis. CO levels from such patients have been reported to persist in refrigerated sealed liquid blood samples stored in the presence of preservatives for at least three years; however, no data exists regarding the persistence of CO in aged, dried bloodstains. It has been noted that the bloodstains on the Shroud of Turin appear too red in color for blood that is reportedly some 600-2000 years old. Various explanations have been put forth for the blood color on the Shroud, including the suggestion that CO bound to hemoglobin via endogenous or exogenous mechanisms is responsible. In this report, the effect of CO exposure on bloodstain color is evaluated and its relevance to the appearance of the Shroud of Turin bloodstains is discussed.

Introduction

Fresh oxygenated blood is bright red in appearance, which upon drying changes into a dark brownish color within approximately 12-24 hours. The molecular basis for the color change in drying blood is well established. When hemoglobin molecules present in red blood cells contain bound oxygen (Hb-O_2), the blood maintains its red color. During the drying process and exposure to air, hemoglobin becomes deoxygenated, and converts to methemoglobin (Hb-Met), giving bloodstains a brownish, black appearance (1-3). The methemoglobin form of hemoglobin is unable to bind oxygen, and is referred to as the oxidized form. Oxidation of hemoglobin is characterized by conversion of iron from the Fe^{2+} state to the Fe^{3+} form (1-3).

Carbon monoxide (CO) is very efficient at binding to hemoglobin, having an affinity that is 250-300 times greater than that of oxygen (4-6). Formation of the hemoglobin-CO (Hb-CO) complex results in a conformation change, with a characteristic absorption spectra in the visible range (7-8). In fresh blood, as Hb-CO concentrations increase the blood takes on a cherry red appearance, a condition that is associated with CO poisoning. As noted by Simini, the term "cherry red" is somewhat subjective as there are numerous species of cherries, each with its own characteristic shade of red and coloration (9). CO levels in blood drawn from patients suffering from CO poisoning are relatively stable, and have been reported to stay constant for up to at least three years when stored in closed tubes containing anti-coagulants (10-12). No information is available, however, regarding the persistence of CO in

aged, dried bloodstains. Thus, it remains to be determined if the "cherry red" color of blood exposed to CO endures over time.

Multiple individuals that have examined the bloodstains on the Shroud of Turin in person have commented on their "too red" color (3). In 2001, Baima Bollone proposed that the red color of the Shroud blood could be explained by the high affinity binding of CO to hemoglobin, occurring as a natural product of hemolysis and the generation of bilirubin (13,14). In this model, the binding of endogenous CO to hemoglobin would persist in bloodstains over time, maintaining the overly red color. Alternatively, it has been suggested that exogenous CO generated during one of the fires in the Shroud's history is the source of CO binding to hemoglobin that could be responsible for the bloodstain color (15). In the current study, the influence of CO exposure on the color of bloodstains is examined and the relevance of the findings to the Shroud of Turin in discussed.

Materials and Methods

Blood Samples-Blood samples were obtained by finger stick and 5-7 drops were applied to parafilm for each experimental group. All work was concluded within 7-10 minutes from the time of initial blood draw preceding the coagulation process. No preservatives were used in any of the studies. Approximately 100-200 µl of blood was transferred to microcentrifuge tubes for each group and CO streamed directly into the tube with gentle mixing. Blood was then transferred onto filter paper or linen and allowed to dry. Alternatively, samples were transferred from parafilm to filter paper or linen first, and CO streamed directly onto the sample.

Generation of CO-In our initial studies CO was produced by the combination of formic acid and sulfuric acid as previously described (16,17). CO gas was collected in syringes and streamed over the sample or in some cases streamed directly through the tubing during gas generation. Alternatively, CO was delivered from a CO source tank (Airgas) through tubing directly onto the sample. Samples were exposed to CO at a flow rate of 5 psi for approximately 60 seconds. All work involving CO by both methods was done in an operational certified fume hood.

Spectroscopy Studies of Dried Bloodstains-Samples were excised from filter paper or linen, cut into small pieces, and suspended in solubilization buffer (0.1M Tris Borate, 0.1% Tween) for approximately 60 minutes. Multiple dilutions of each sample were made in distilled water and samples read using a PASCO 2600 portable spectrometer. Controls included filter paper or linen without blood subject to the same procedure. In certain experiments, untreated fresh blood or CO-treated blood was added immediately to solubilization buffer, diluted in distilled water as above, and analyzed.

Results

The typical color change in dried blood over time, changing from red to brown is shown in Figure 1 (Figure 1). To allow the samples to be photographed at the same time and under

the same conditions, blood was drawn over a 12 day period, counting down backwards to time zero. This illustrates the characteristic conversion of fresh oxygenated red blood to

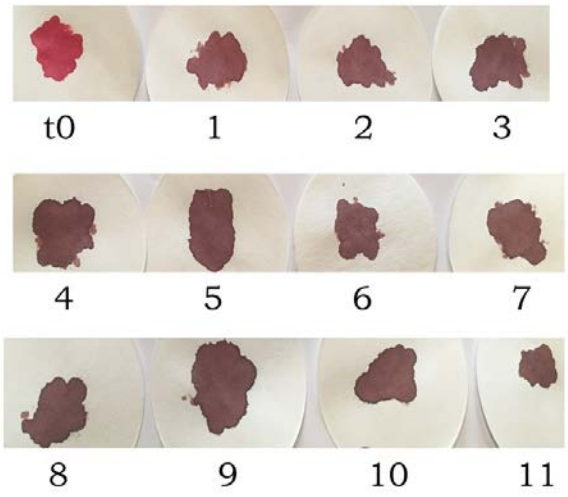

Figure 1. Time course of color change of normal, untreated blood. *Blood samples obtained by finger stick were blotted onto filter paper and allowed to dry for the time period indicated (in days). See text for details. The data shown are representative of at least three separate experiments.*

methemoglobin, a color change that is familiar to most having encountered the transitional appearance of bloodstains during normal, everyday circumstances.

Effect of CO on bloodstain color part 1: generation of CO by acid mixture

To study the effect of CO on the appearance (color) of bloodstains, CO was initially generated by mixing formic acid and concentrated sulfuric acid with mild heating in the reaction: $HCOOH + H_2SO_4 \rightarrow CO + H_2SO_4 \cdot H_2O$ as described (16,17). This is a common method used in the production of CO, particularly in instructional laboratories to medical and graduate students. (Note: Any such studies must be performed in a fume hood). CO was applied to freshly obtained blood in two ways: Either CO was streamed into a microfuge tube containing blood, and blood subsequently transferred to filter paper (Figure 2, top), or blood was transferred to the filter paper first and then CO was streamed directly above the bloodstain (Figure 2, middle). As demonstrated, exposure of blood to CO in either protocol resulted in a notable color change, having a relatively brownish appearance compared to the control sample (Figure 2). Similar results were obtained when linen was used (Figure 2, bottom). To allow an easier comparison, Figure 3 shows an experiment in which blood was first transferred to filter paper and divided in two; half of the sample was treated with air, the other half with CO. Following treatment, each half was then placed adjacent to each other (Figure 3). Interestingly, the resultant color change after CO exposure appeared distinct from fresh blood, having an appearance more similar than that of aged blood (brownish) than a "cherry red" coloration (Figures 2 and 3).

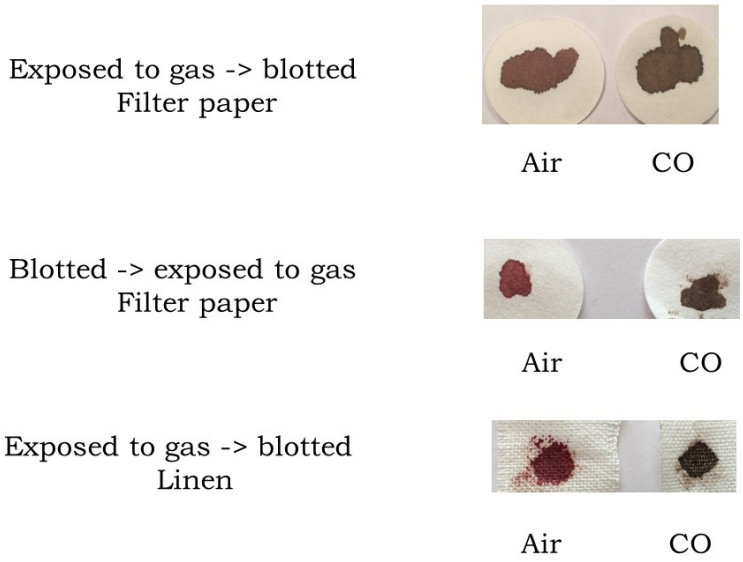

Figure 2. Effect of CO on bloodstain color (acid mixture). *Blood samples obtained by finger stick were exposed to gas and then blotted onto filter paper (top panel); alternatively, samples were blotted onto filter paper and then CO was immediately streamed directly onto the bloodstain (middle panel). The bottom panel uses a similar approach to that shown at the top except that linen was used. See text for details. The data shown are representative of at least three separate experiments.*

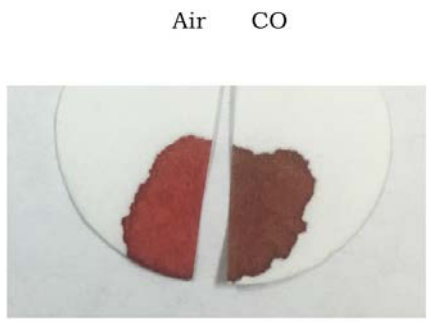

Figure 3. Side by side comparison of the effect of CO on bloodstain color (acid mixture). *Blood samples obtained by finger stick were blotted onto filter paper, divided in half, and immediately exposed to gas. The sample on the left was exposed to air; that on the right to CO. See text for details. The data shown are representative of at least three separate experiments.*

Because the method that was used to produce CO gas involved the combining of two acids, there was concern that the observed coloration might be related to a pH effect. To examine this possibility, pH indicator paper was placed inside the gas collection syringe and also attached to the filter paper adjacent to the bloodstain as CO was streamed above it. In both cases, a prompt drop in pH (from 7 to approximately 2) was noted as exposure to the products began to occur (Figure 4). When the same protocol was followed using formic acid (HCOOH)

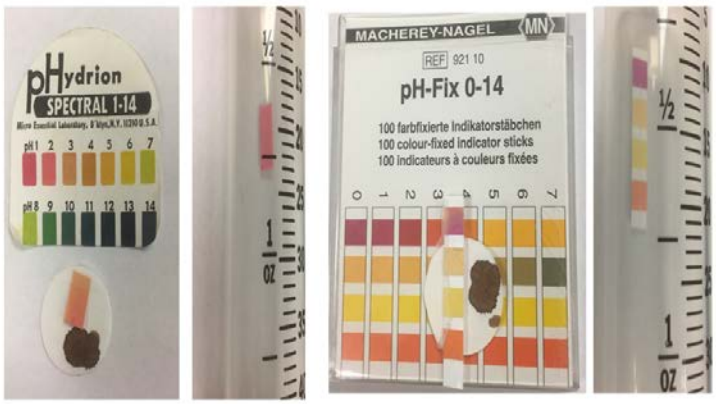

Figure 4. Generation of products in the reaction mixture results in a change in pH. *CO gas was generated using the acid mixture protocol and streamed over bloodstains to which two different types of pH indicator paper were attached. Additionally, pH indicator paper was included in the gas collection syringe. Upon exposure to gas, a drop in pH was observed from approximately 7 to 2-3. No pH change was observed with each reactant was singularly used in the protocol (data not shown); see text for details. The results shown are representative of at least three separate experiments.*

or sulfuric acid (H_2SO_4) alone, gas production did not occur and no color change took place (data not shown). Thus, these results were specific for the products of the mixture. Further experimentation on the effect of pH in the absence of CO indicated that there was a substantial influence in both bloodstain appearance and properties across a pH range of 1-12 (Kearse, unpublished observations). While the effect of pH has been hypothesized to influence the appearance of the Shroud bloodstains, (15) such results are beyond the scope and intent of the current study and are not discussed further, being treated as work in progress. Most importantly, the current results indicate we were unable to use this particular experimental set up in our studies as it introduced an additional variable that could influence bloodstain color.

Effect of CO on bloodstain color part 2: Using purified CO gas

These studies were continued using purified CO purchased from a local gas supply company. Such an approach was not used initially both due to expense and because the previous experimental set up is frequently cited as a relatively easy method for production of CO in the laboratory. As shown in Figure 5, when the experiments were repeated using purified CO, the results were markedly different than before. CO-treated samples exhibited a color change that corresponds more with what has been described for liquid blood (Figure 5a), having a "cherry red" type appearance. To the author, it appeared as a darker, richer red similar to the coloration of the Bing cherry variety. A similar color change was observed when samples were blotted onto a variety of material, including cotton (Q-tips), and modern linen, both unbleached and natural (Figure 5b).

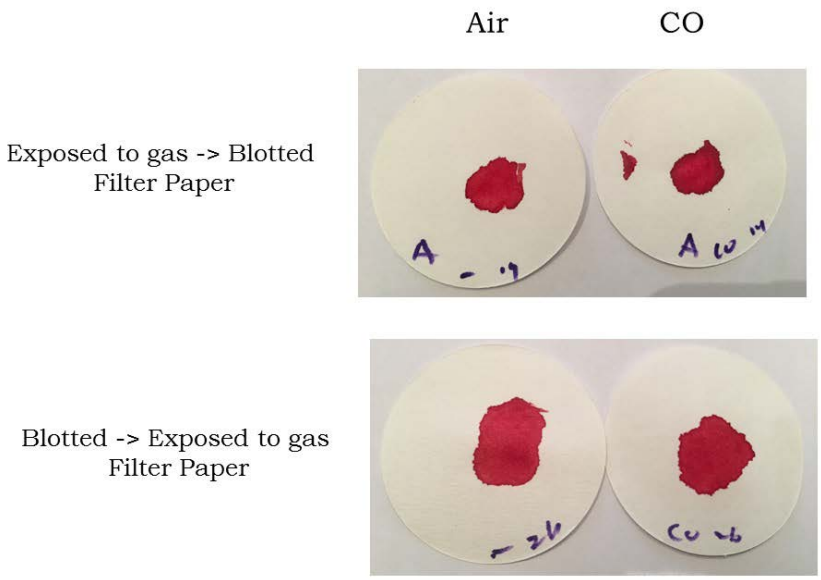

Figure 5a. Effect of CO on bloodstain color (purified gas). *Blood samples obtained by finger stick were exposed to gas and then blotted onto filter paper (top panel); alternatively, samples were blotted onto filter paper and then CO was immediately streamed directly onto the bloodstain (bottom panel). See text for details. The data shown are representative of at least three separate experiments.*

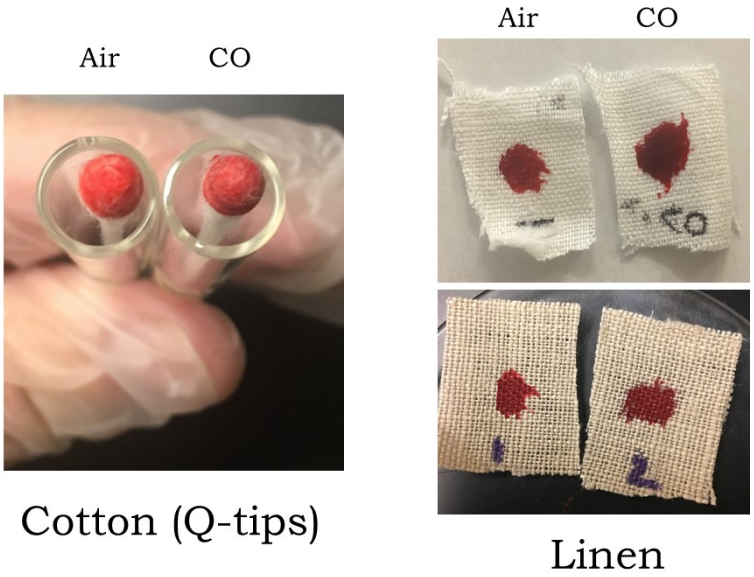

Figure 5b. Effect of CO on bloodstain color (purified gas). *Blood samples obtained by finger stick were exposed to gas and then blotted onto cotton (left panel), modern bleached linen (top right panel) or modern natural linen (bottom right panel). See text for details. The data shown are representative of at least three separate experiments.*

Immediately following exposure, samples were also examined by spectrometry. As shown in Figure 6, spectral analysis of fresh blood showed an absorbance pattern in agreement with the reported profile for fresh blood containing Hb-O_2, showing the major Soret band at ~420 nm and the oxyhemoglobin β band and oxyhemoglobin α band at ~540 and ~575 nm, respectively, (Figure 6a), (18). When untreated and CO- treated samples were analyzed, CO treatment resulted in a profile shift consistent with what has been previously demonstrated (Figure 6b), (7,8). Similar results were obtained whether

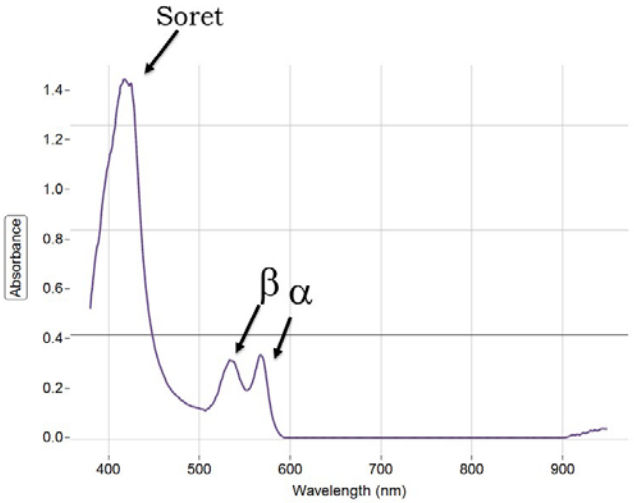

Figure 6a. Spectral analysis of fresh blood. *Blood samples obtained by finger stick were analyzed by spectroscopy as described in Materials and Methods. These results show the three major bands characteristic of freshly obtained, oxygenated blood. The data shown are representative of at least three separate experiments.*

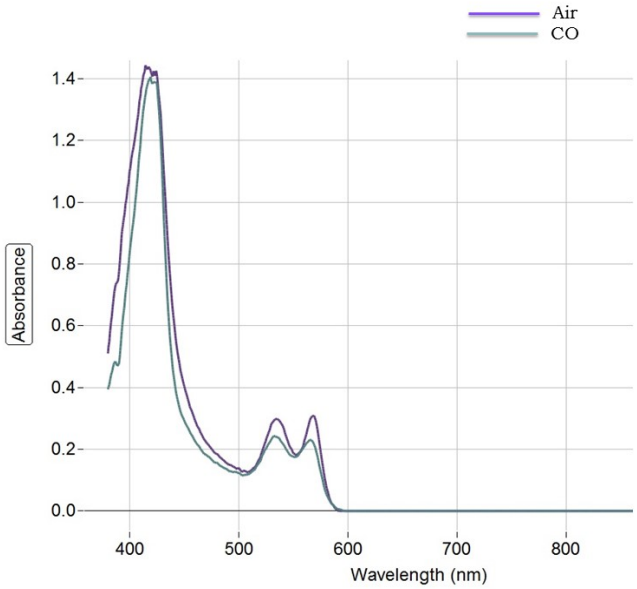

Figure 6b. Spectral analysis of control versus CO-treated blood. *Blood samples obtained by finger stick were exposed to air or CO and analyzed by spectroscopy as described in Materials and Methods. The data shown are representative of at least three separate experiments.*

samples were treated with gas and then immediately analyzed or treated with gas, blotted to filter paper, and extracted prior to analysis (see below). When CO-treated samples were examined several days after treatment, no difference was observed visually relative to untreated control groups (Figure 7a) and the absorbance profiles were essentially the same

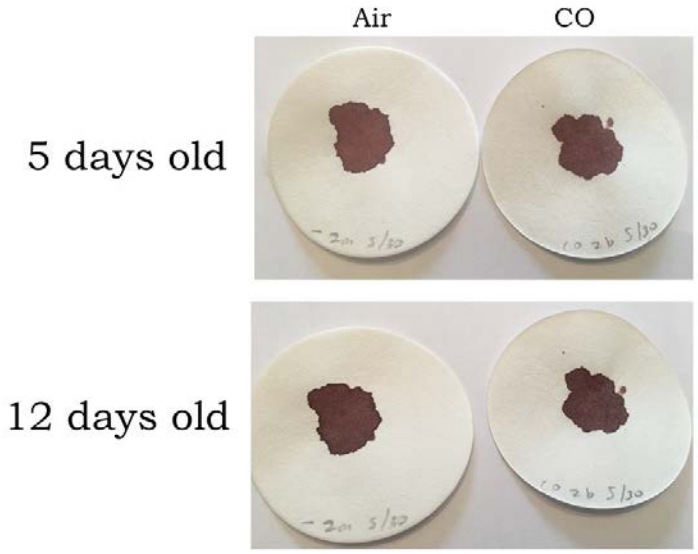

Figure 7a. Effect of CO on bloodstain color does not persist with aging. *Control and CO-treated samples were aged for the time period indicated. See text for details. The data shown are representative of at least three separate experiments.*

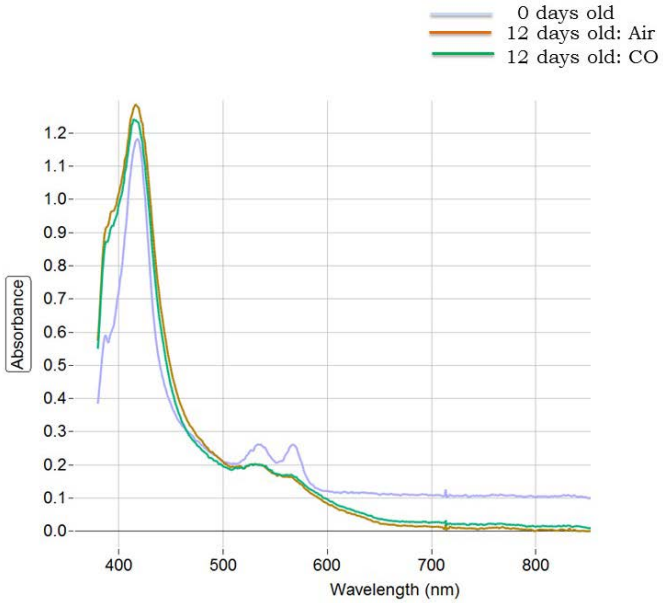

Figure 7b. Spectral analysis of control and CO-treated aged bloodstains. *Samples were transferred to filter paper, aged for the time period indicated, and analyzed by spectroscopy as described in Materials and Methods. Control samples show a shift in absorbance profiles upon aging, consistent with the conversion of oxyhemoglobin to methemoglobin. CO-treated samples show an identical absorbance profile as control samples. The data shown are representative of at least three separate experiments.*

(Figure 7b). These data indicate that like control bloodstains, CO-treated bloodstains undergo conversion to Hb-Met with similar kinetics. Finally, untreated aged bloodstains were divided in half as before to evaluate if exposure to CO might alter their appearance. As demonstrated, no visual difference was observed between untreated and CO-treated samples, either visually (Figure 8a) or in spectral analysis (Figure 8b). Taken together, these results show that exogenous CO does not affect the color of aged bloodstains.

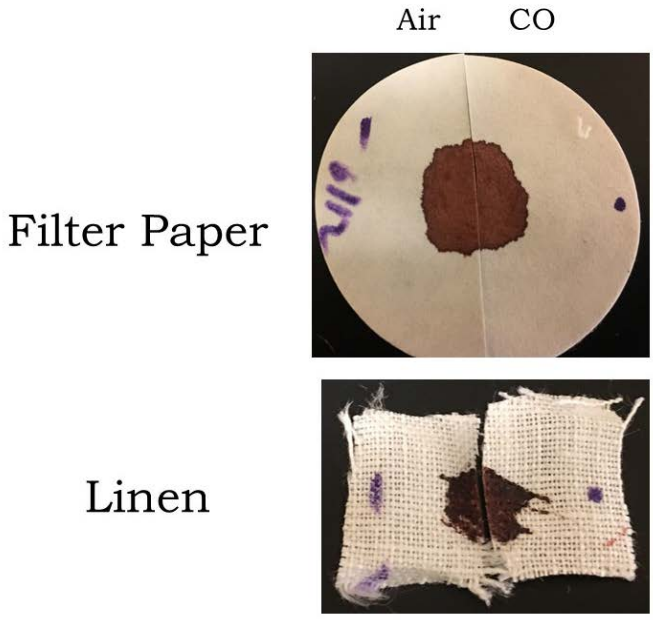

Figure 8a. Side by side comparison of aged control versus CO-treated bloodstains. *Twelve days old bloodstains were divided in half, and exposed to either air or CO. Samples were then placed side by side for visual comparison. See text for details. The data shown are representative of at least three separate experiments.*

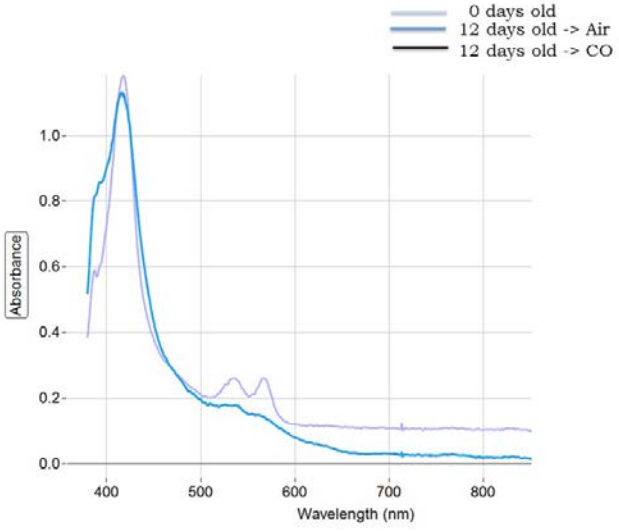

Figure 8b. Spectral analysis of control and CO-treated aged bloodstains. *Twelve days old bloodstains were exposed to air or CO, extracted, and analyzed by spectroscopy as described in Materials and Methods. The data shown are representative of at least three separate experiments.*

Discussion

The current report has evaluated the effect of CO exposure on the appearance of bloodstains, particularly their conversion from red to brown upon aging. Somewhat surprisingly, our initial results indicated that the protocol often used for the generation of CO in the laboratory for instructional purposes (16,17) had an accompanying pH effect which confounded the interpretation of our initial findings. To circumvent this issue, we switched to using purified CO that was delivered from a pressurized tank. These results show that CO exposure affects the color of blood which immediately persists when applied to various matrices such as filter paper, cotton or linen. CO treatment was able to affect a similar color change on fresh bloodstains applied to these mediums if CO was added as they were drying. Importantly, this color change did not persist over time as control and CO-treated bloodstains were similar in appearance after several days. The accompanying spectroscopy data corroborated these findings. These results argue against the hypothesis originally presented by Baima Bollone that endogenous CO, generated during the physiological breakdown of red blood cells and bilirubin production, is responsible for the reddish appearance of the Shroud of Turin bloodstains (13). In Adler's spectroscopic studies on Shroud blood samples, he reported that the major species detected was that of acid methemoglobin, although certain undetermined peaks were present (19). It is reasonable that Hb-CO would not persist in dried samples exposed to air as the concentration of oxygen is much greater, which is predicted to exchange and result in normal conversion to Hb-Met. Our studies also examined the suggestion that exposure of bloodstains to exogenous CO generated during the event of a fire might result in their "regeneration" to a reddish color. As shown in the current report, this appears very unlikely as no change was observed even when CO was directly streamed onto aged samples. Moreover, as aged blood exists in the Hb-Met form, it is unclear as to how such CO binding to Hb-Met might occur as this species is conformation different than native Hb.

Summary

In summary, these data demonstrate that CO treatment alters the color of fresh blood as it is drying but that the enhanced redness does not persist over time when such samples are exposed to air. Our findings also show that aged blood samples cannot be rejuvenated to a reddish color following exposure to CO. Thus, our results argue against any contribution of CO in the color and appearance of the bloodstains on the Shroud of Turin.

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