# Hemolysis, bilirubin, and the color of the bloodstains on the Shroud of Turin

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## Abstract

Numerous circumstances may affect the appearance of aged bloodstains, including blood concentration, environmental conditions, and the type of substance on which they dried. A frequent observation of those that have examined the Shroud of Turin is that the bloodstains are more reddish than would be expected for aged blood. The reddish color is not believed to be due to paint; but rather, has been proposed to result from a property of the textile or the blood itself. Here, I have examined two major hypotheses related to the reddish color of the Shroud bloodstains: (i) the effect of hemolytic agents and (ii) elevated bilirubin levels, about which relatively little information exists, even for normal blood. These data show that bloodstain color is unaffected by treatment of textiles with various hemolytic agents, including saponin, and that a reddish color does not persist in bloodstains containing high amounts of bilirubin. In addition, I noted an unexpected effect of glycerin-treated fabric on bloodstain appearance. The contributions of these studies to the reference data available for bloodstain evaluation and their implications for the Shroud of Turin are discussed.

**Keywords: bloodstains, hemolysis, bilirubin, saponin, glycerol, Shroud of Turin**

## Introduction

In aged objects that are of unknown origin, evaluation of bloodstains can be particularly challenging relative to those that contain certain source material to help provide a context. The Shroud of Turin, or Turin Shroud (TS), is an approximately 14 x 3.5 feet linen cloth bearing the faint frontal and dorsal images of a man with reddish areas corresponding to wounds at the head, hands, feet and back. The first endeavor to scientifically evaluate the nature of the TS bloodstains began in 1973 by members of a "commission of experts", led by Frache et al. Their results were negative, hampered by ineffective solubilization methods, with the conclusion that "the negative answer to the investigations conducted does not permit an absolute judgement of the hematic nature of the material under examination" [1,2]. In 1978, the bloodstains were evaluated again as part of the STURP (Shroud of Turin Research Project) examination, involving over forty scientists of various disciplines; samples were also collected independently by Bollone and colleagues at this time. To date, this examination remains the most thorough investigation of the cloth, and is the main depository of the data related to the characterization of the bloodstains. It was concluded from such studies that the TS bloodstains were composed of real blood components and not pigment or dyes [2-9]. Most recently, spectroscopic analysis has shown the bloodstains consist of methemoglobin, the deoxygenated form of hemoglobin expected for aged blood, corroborating previous findings on the hemoglobin species present [3,10].

One unusual feature of the TS bloodstains is that their color is more reddish than would be expected for aged blood, which typically appears brownish to black. Rogers proposed that treatment with Saponaria (soapwort) was part of the normal processing of ancient linen, leaving a surface residue on the TS that retained hemolytic activity. He noted that blood added to Saponaria-treated cloth maintained a reddish color some thirty years later, compared

to controls which were black [11]. Unfortunately, no data or pictures from these experiments have ever been published. Alternatively, it was suggested that high amounts of bilirubin present in blood exudates are responsible for the reddish color of the TS bloodstains, resulting from heightened hemolysis *in vivo* due to excessive trauma; lysis of red blood cells would result in release of hemoglobin, which, in turn, would be converted into bilirubin [2,6,12]. Adler maintained that a brown color (oxidized hemoglobin, i.e. methemoglobin) plus yellow/orange (bilirubin) would result in a reddish appearance. Moreover, he indicated that reddish color could be recreated in an *in vitro* blood simulacrum containing high bilirubin, although unfortunately no data or pictures from these experiments were ever published [2,6,12].

In the initial consideration of these hypotheses, I found that little background information existed regarding the effects of hemolysis or elevated bilirubin levels on the color of bloodstains overall. While a few studies have been done related to the influence of bilirubin on TS bloodstains [10,13,14], several caveats were present; specifically: i) anti-coagulants were included; ii) bilirubin levels were in the range of 5-10x above normal (Adler would propose in his hypothesis that the concentrations would be significantly higher); and iii) multiple forms of bilirubin were present (see below). The purpose of the current study was twofold: to contribute to the database of endogenous and environmental factors that might affect bloodstain appearance, in general, and to apply such results to the existing knowledge of the TS.

**Materials and Methods**

**Linen and filter paper**. Hanks of natural, unprocessed flax (Vavstuga, Shelburne Falls, MA) were woven in a 3:1 herringbone pattern with a Z twist, similar to what has been described for the TS, by professional weaver Tess Farley (USA). Filter paper sources were Whatman filter paper 1mm and 3mm (Whatman, Amazon.com, USA).

**Blood**. Human blood was obtained from healthy volunteers by the finger stick method using a Health Lancing device (CVS pharmacy, USA) fitted with a microlancet (CVS Pharmacy, USA). Blood was added to parafilm "M" laboratory film (Bemis, Amazon.com, USA) and transferred to filter paper or linen using a micropipet. Approximately 10-20 microliters of blood was used for each group. For freeze-thaw treatment, whole blood was placed in the freezer (-20$^{o}$C) overnight, removed to thaw, and this cycle repeated 1-2 more times. Whole blood and serum from wild type and Gunn rats was obtained from RRRC, University of Missouri, USA. The Gunn rat contains a spontaneous mutation in the UDP-glucoronosyltransferase (ugt) gene and expresses high levels of bilirubin, approximately 7x that of normal, all in the unconjugated form [15]. Whole blood and serum from wild type and hugt -/- mice was kindly provided by Drs. Robert Tukey and Nghia Nguyen, University of California, Sand Diego, USA. In ugt -/- mice, the UDP-glucoronosyltransferase gene has been genetically disrupted; these mice express extremely high (lethal) levels of bilirubin. In hugt -/- mice, the UDP-glucoronosyltransferase gene has been genetically disrupted and replaced with a human counterpart; these mice express high levels of bilirubin, approximately 10-12x above normal [16].

**Saponin preparation and treatment of textiles**. Four sources of saponin were used in these studies: extracts from soapwort roots and leaves, prepared according to the methods of Budan et al. [17]; saponin on an applicator stick, obtained from In His Hands Birth Supply company (Liberty Hill, TX); and saponin solution (in glycerol, water) purchased from HawaiiPharm, HI through amazon.com. For pre-treatment experiments, filter paper or linen was soaked overnight in saponin solution or solvent alone (control), removed and allowed to dry. In experiments using saponin on an applicator stick, saponin was mixed directly with the cell preparation prior to addition to linen or filter paper. Triton X-100 detergent was purchased from amazon.com (USA) and used at a final concentration of 1%.

**Hemoglobin release assay.** Following treatment, phosphate buffered saline was added, samples were spun in a microcentrifuge, supernatants removed, and transferred to a new tube; supernatants were analyzed using a GENESYS 20 spectrophotometer at an absorbance of 543, as described by Rodi et al. [18].

**Bilirubin preparation**. Unconjugated and conjugated bilirubin (Cayman Chemical Co., Ann Arbor, MI) were resuspended in chloroform or water, respectively, at a concentration of 10 mg/ml. The reported average range for total bilirubin is 0.2-1.0 mg/dL; with levels in healthy individuals of ~ 0.7 mg/dL and 0.3 mg/dL for unconjugated and conjugated bilirubin, respectively. For these studies, a normal level for total bilirubin was taken at 1.0 mg/dL, and exogenous bilirubin added to whole blood for a final concentration of 100-500 mg/dL. Control groups received solvent alone at the equivalent volume.

**Chemical induction of methemoglobin**. $NaNO_2$ treatment of whole blood or lysates to induce methemoglobin formation was performed according to the method of Patton et al. [19], and was typically used at a concentration of 50 mM. Methemoglobin formation was verified by spectrophotometry using a PASCO 2600 portable spectrometer.

## Results

### Effect of hemolysis on bloodstain color

The effect of hemolysis on bloodstain color was examined in two different ways, first by adding blood to material that had been treated with saponin extract, prepared from roots or leaves from the soapwort plant; and second, addition of red blood cell lysates prepared by detergent (saponin) or non-detergent methods (freeze-thaw). As demonstrated in Figure 1, blood was oxidized to a similar brown color on linen that had been pre-treated with saponin extracts, as that of untreated linen [Figure 1]. Relatedly, when cells were lysed using saponin on an applicator stick or by freeze-thaw methods, and then added to untreated material, a reddish color did not persist over time [Figure 2]. Similar results were observed when saponin sticks were solubilized in distilled water and the solution used to pre-treat linen or filter paper (data not shown). The effectiveness of saponin in induction of hemolysis was evaluated by hemoglobin release, using spectroscopy as described by Rodi et al. [18]. Extracts from saponin roots were 94% as effective as Triton-X 100 detergent in hemolysis induction, which was used as the standard (set at 100%) in this assay [Figure 3,A]; saponin

leaves were less effective, at 65% [Figure 3, A]. When cells were lysed using saponin on an applicator stick or freeze-thaw methods, these were 75% and 95% as effectual as Triton-X 100, respectively [Figure 3,B]. Taken together, these data show that hemolysis is not associated with the persistence of red color in bloodstains over time.

Interestingly, unlike previous results, when a fourth source a saponin was utilized, a reddish color was maintained in bloodstains [Figure 4]. This particular saponin (Saponin 4) was purchased in an aqueous form, containing ~ 40% glycerol. Most importantly, a similar color effect was observed in groups that were treated with glycerol in the absence of saponin [Figure 5a]. The reddish color with glycerol treatment persisted for at least 1 week, but was not observed after several months [Figure 5b]. Collectively, these results indicate that the color results observed with this specific saponin solution, Saponin 4, can be explained as a nonspecific consequence of glycerol (glycerin).

**Effect of bilirubin on bloodstain color**

Next, I examined the effect of bilirubin on bloodstain color, which as previously mentioned relies on Adler's premise that a brown color (methemoglobin) plus yellow/orange (bilirubin) would result in a reddish appearance. Bilirubin exists in two forms in the body, an unconjugated form which is water-insoluble, and a conjugated form which is soluble; conjugation is necessary for bilirubin to be effectively excreted from the body. Adler would particularly favor the unconjugated form of bilirubin being in excess, due to the rapid occurrence of hemolysis resulting from a proposed excess of physiological trauma [2-4]. Methemoglobin (deoxygenated hemoglobin) is formed during the natural aging of bloodstains, but may also be rapidly induced by treatment with $NaNO_2$ [17]. As shown in

Figure 6, $NaNO_2$ treatment effectively oxidized blood to a brownish color [Figure 6]; this was observed when either whole blood or lysates were used [Figure 7]. The presence of methemoglobin following $NaNO_2$ treatment was verified by spectrometry analysis (data not shown).

As shown in Figure 8, bloodstains in control and $NaNO_2$-treated samples containing 100 times normal levels of bilirubin (in the unconjugated form) were comparable in appearance [Figure 8]. Most importantly, when blood containing high levels of unconjugated bilirubin was aged naturally (without any $NaNO_2$ treatment), a brownish color was observed with time, similar to control groups [Figure 9]. Identical results were obtained when comparable levels of conjugated bilirubin were used [Figure 10], or when samples contained a mixture of both bilirubin forms [Figure 11]. Bilirubin was also used at lower (10x) and higher (200x, 500x) levels, but in no instance did a reddish color continue over time ( (data not shown). Taken together, these data demonstrate that reddish color does not persist in bloodstains containing high amounts of bilirubin, present in either unconjugated or conjugated forms.

Finally, in extension of these results, experiments were also performed using blood from rodent strains that contain endogenously high levels of bilirubin *in vivo*, specifically the Gunn rat and ugt -/- mice. The Gunn rat contains a spontaneous mutation in the gene responsible for bilirubin conjugation, with hyperbilirubinemia, all in the unconjugated form. In ugt knockout mice (ugt -/-) mice, the conjugation gene has been intentionally deleted, such mice have a very finite life span (days to 1 week). Hugt-/- mice are knockout mice with a human copy of the conjugation gene, which still exhibit hyperbilirubinemia at a young age. In agreement with our previous results using human blood with exogenous bilirubin added *in*

*vitro*, a reddish color did not persist in bloodstains from rodent strains with hyperbilirubinemia [Figures 12,13]..

**Discussion**

The current study represents the first examination of the hypothesis proposed by Rogers that hemolysis resulting from a surface residue of saponin is responsible for the reddish color of the TS bloodstains. These data demonstrate that bloodstain color was not affected by various hemolytic agents, including saponin obtained from multiple sources. Rogers suggested the surfactant saponin was responsible due to its reported use in ancient linen manufacturing; however, if the reddish color of the bloodstains on the TS were specifically due to the action of a hemolytic agent, various lysis methods should yield a similar result. As demonstrated in this report, hemolysis was not associated with persistence of red color in bloodstains.

In the one case where saponin appeared to affect bloodstain color, this was shown to be a nonspecific effect due to the presence of glycerol (glycerin). Glycerol is used in the freeze preparation of red blood cells [20,21], and can induce hemolysis at room temperature, although this is the first instance that I am aware of in which a color effect on bloodstains has been noted. The color effect could be an important consideration in the investigation of homicides where the victim had recently washed with a hi-glycerin product, or residual towels were used in the cleanup of bloodstains afterwards. Unlike experimental results, where the starting point may be documented for comparison, such data may not be obtainable in various crime scenes, making the initial evaluation of bloodstain appearance (color) somewhat subjective. Bloodstains on fabrics containing residue from hi-glycerin soaps

might seem more recent due to their reddish color. In future studies it will be interesting to determine if treatment of textiles with other related compounds shows a similar effect on bloodstain appearance.

A second hypothesis related to the TS was evaluated, that the reddish color of bloodstains is related to high levels of hemolysis, methemoglobin, and bilirubin. A few reports related to the TS have examined the effect of bilirubin on bloodstain color; however, as noted in the introduction, such studies were limited by several constraints. The current results importantly extend these previous studies by: i) using blood without anti-coagulants; ii) including much higher levels of bilirubin; and iii) specifically focusing on the various forms of bilirubin that were present. I found that increased bilirubin levels did not result in the persistence of the reddish color of bloodstains over time, regardless of which type of bilirubin predominated. These results argue against elevated bilirubin levels being responsible for the reddish color of bloodstains on the TS, and provide evidence that high bilirubin content, in general, does not affect their appearance of bloodstains over time.

Why are the Shroud's bloodstains reddish in color? The answer remains unsolved, although using blood from a jaundiced patient and continuous wave ultraviolet (uv) irradiation, Lascio and colleagues have recently suggested that a combination of high bilirubin/uv exposure generates a color shift that is still measurable after four years [10]. Although in Adler's studies on the TS, no estimation of specific bilirubin levels/amounts was given, the suggestion that that high bilirubin levels exist in TS bloodstains is interesting as bilirubin is notably light sensitive. Whether or not the stability of bilirubin in bloodstains is dissimilar, particularly over many years, is unclear.

Adler's hypothesis is based on the suppositions that the TS bloodstains are the result of the cloth being wrapped around a body, a body that had undergone severe trauma within the previous twenty-four hours, resulting in increased levels of endogenous bilirubin. As noted in the introduction, a paucity of information exists regarding the correlation of bodily suffering with alterations in bloodstain color, although in fairness, this may be an issue that has not received much prior attention. Several years ago, Lee and colleagues made the interesting observation that bilirubin is also found in the red-orange fruits and flowers of certain plants, which is chemically indistinguishable from bilirubin expressed in humans [23, 24]. Some might argue that an artisan may have used a plant with a red-orange pigment to mix with blood for coloration, and unknowingly also added bilirubin (in a relatively concentrated form) in the creation of the TS. It is uncertain if such plant pigments would have been detected in the relatively limited studies that have been performed on TS bloodstains. On the other hand, if bilirubin levels were the result of physical trauma, this could be an important factor in bloodstain evaluation, particularly in relation to victims of severe suffering or torture. Perhaps this is a consideration for forensic investigators and pathologists in the future, to note any anomalies (or not) in the color of aged blood under such circumstances.

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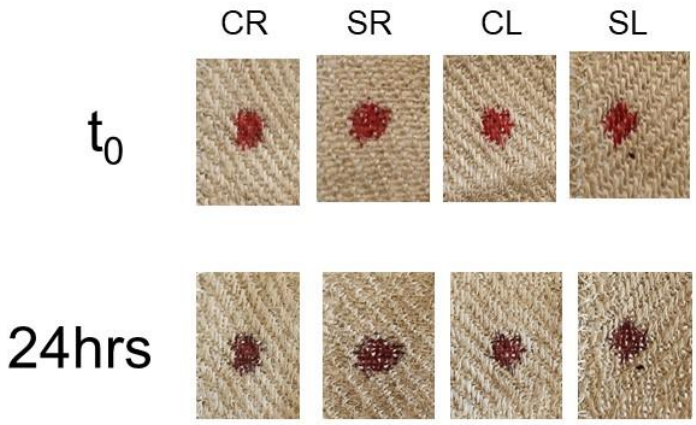


**Figure 1. Bloodstain color on linen treated with saponin extracts.** Linen samples were treated with saponin extracts purified from soapwort roots (SR) or leaves (SL) as described in Materials and Methods. Control extracts (CR, CL) were prepared in parallel and were treated identically as those containing saponin. Whole blood was added to the cloth and color evaluated immediately ($t_0$) or 24 hours later. The data shown are representative of three separate experiments.

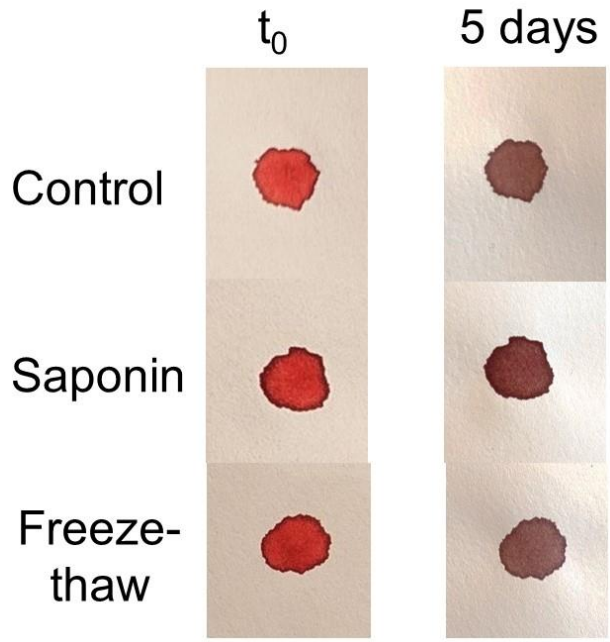


**Figure 2. Effect of hemolysis on bloodstain color.** Whole blood was either untreated (control), treated with saponin on an applicator stick, or subject to several cycles of freeze-thawing to induce hemolysis. Samples were applied to filter paper and color evaluated immediately ($t_0$) or 5 days later. The data shown are representative of three separate experiments.

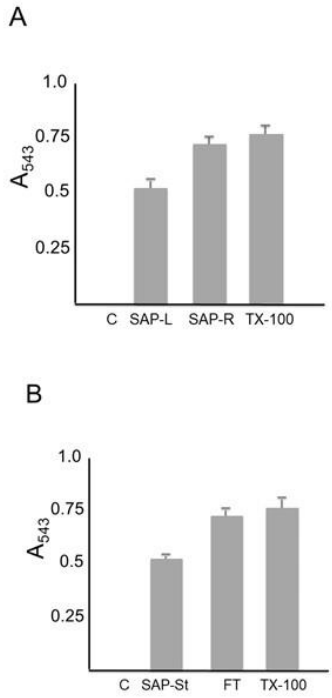


**Figure 3. Hemolysis induction as measured by hemoglobin release.** Samples were treated with the indicated substance and supernatants evaluated at an absorbance of 543 nm to measure release of hemoglobin, indicative of cell lysis. Panel A (Top): C = Control; SAP-L = Extract from saponin leaves; SAP-R = extract from saponin roots; Tx-100 = Triton X-100 detergent. Panel B (Bottom): C = Control; SAP-St = Saponin on an applicator stick; FT = Freeze Thaw; Tx = Tx-100 detergent. Each group was performed in triplicate; data are the mean plus standard deviation of absorbance at 543 nm. The results shown are representative of three separate experiments.

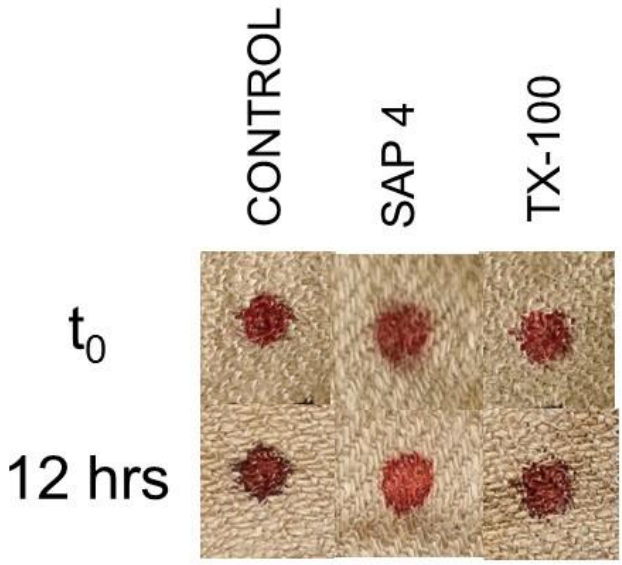


**Figure 4. Effect of saponin solution 4 on bloodstain color.** Whole blood was added to linen pre-treated with the indicated solutions and color evaluated. Control samples were treated with distilled water; SAP 4 is a commercially prepared aqueous solution of saponin. TX-100 = Triton X-100 detergent. The data shown are representative of three separate experiments.

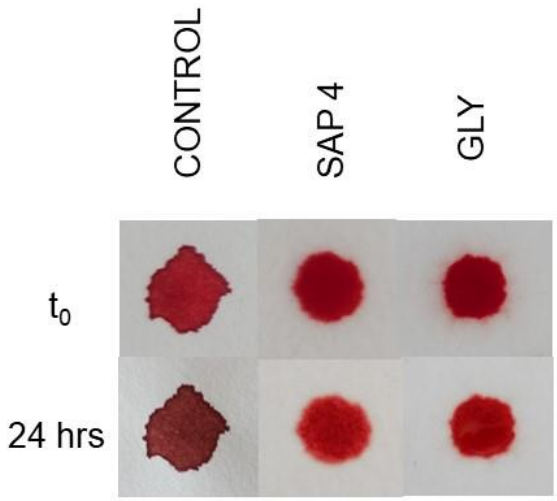


**Figure 5a. Short term effect of glycerol on bloodstain color.** Filter paper was pre-treated with distilled water (control), saponin 4 solution (SAP 4), or 40% glycerol solution (GLY). Whole blood was added and color evaluated at the time period indicated. The data shown are representative of three separate experiments.

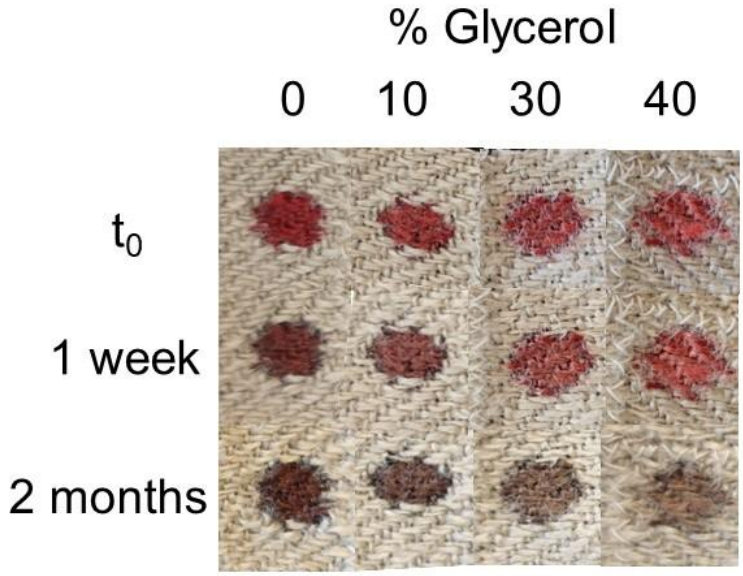


**Figure 5b. Long term effect of glycerol on bloodstain color.** Linen was pre-treated with glycerol solution at various concentrations; whole blood was added and color evaluated at the time period indicated. The data shown are representative of three separate experiments.

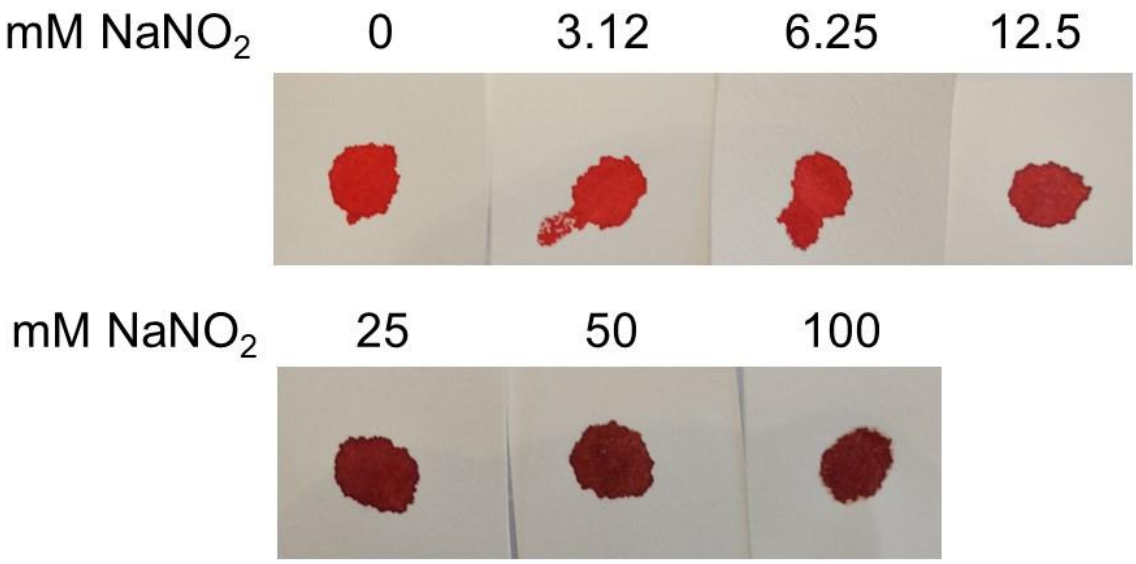


**Figure 6. Dose response treatment of $NaNO_2$ and bloodstain color.** Blood was treated with the indicated concentration of $NaNO_2$ to induce formation of methemoglobin, and transferred immediately to filter paper. The data shown are representative of three separate experiments.

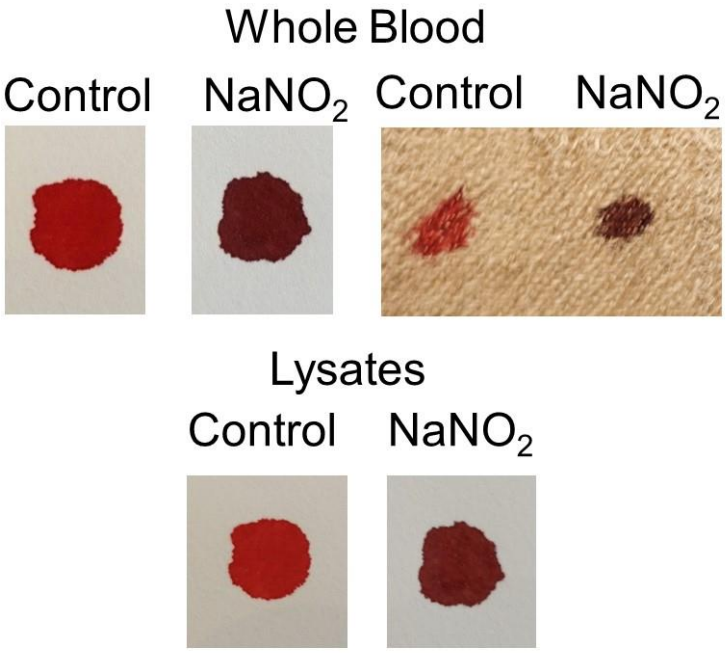


**Figure 7. Effect of $NaNO_2$ treatment on color of whole blood and lysates.** Whole blood (top) or lysates (bottom) were treated with the concentration of $NaNO_2$ indicated, and transferred to material. Color was evaluated immediately. In the groups shown on the top left, filter paper was used, on the top right, linen. In the bottom group (lysates), filter paper was used. The data shown are representative of three separate experiments.

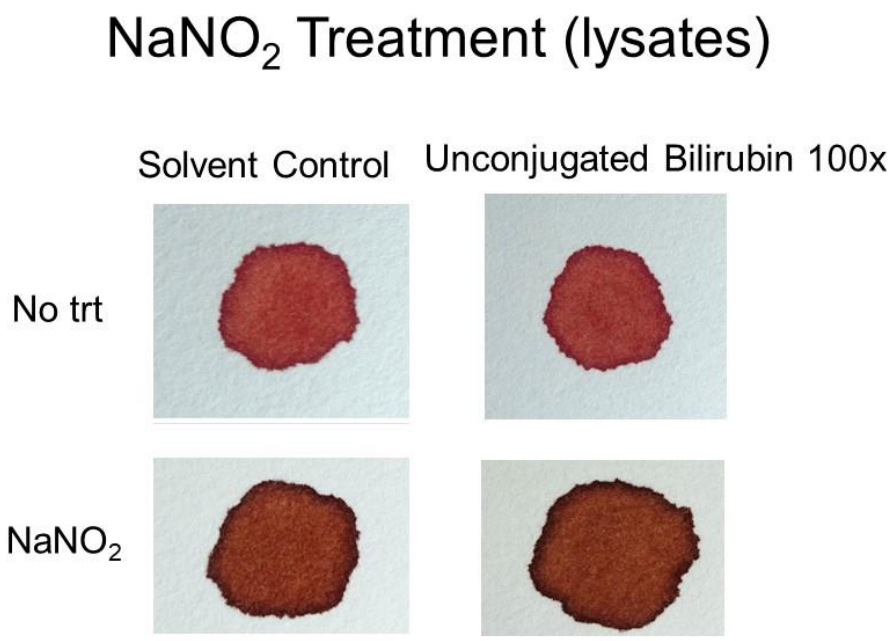


**Figure 8. Effect of $NaNO_2$ treatment plus high bilirubin on bloodstain color.** Lysates were treated with $NaNO_2$ and mixed with unconjugated bilirubin corresponding to 100x normal levels (see Materials and Methods for details), then transferred to filter paper. Color was evaluated immediately. The data shown are representative of three separate experiments.

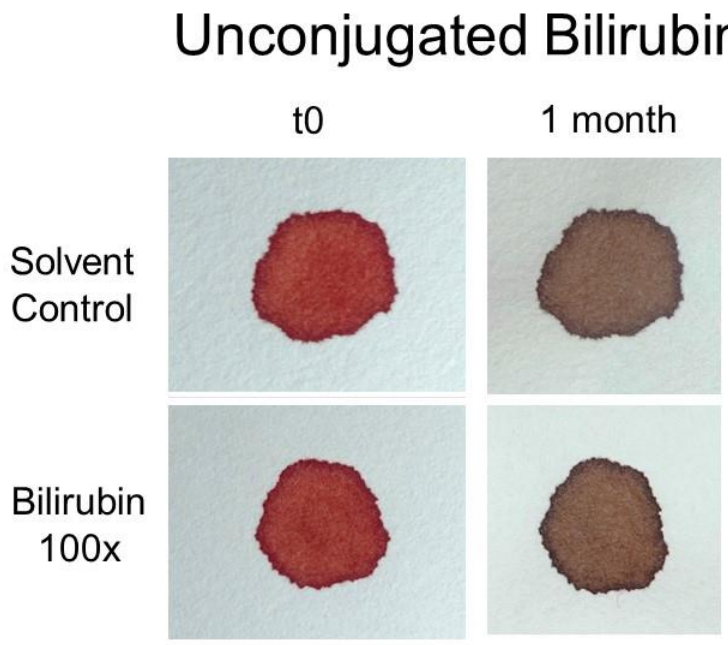


**Figure 9. Effect of high bilirubin (unconjugated) on bloodstain color.** Whole blood was mixed with unconjugated bilirubin corresponding 100x normal levels (see Materials and Methods for details), then transferred to filter paper. Color was evaluated immediately and 1 month later. The data shown are representative of three separate experiments.

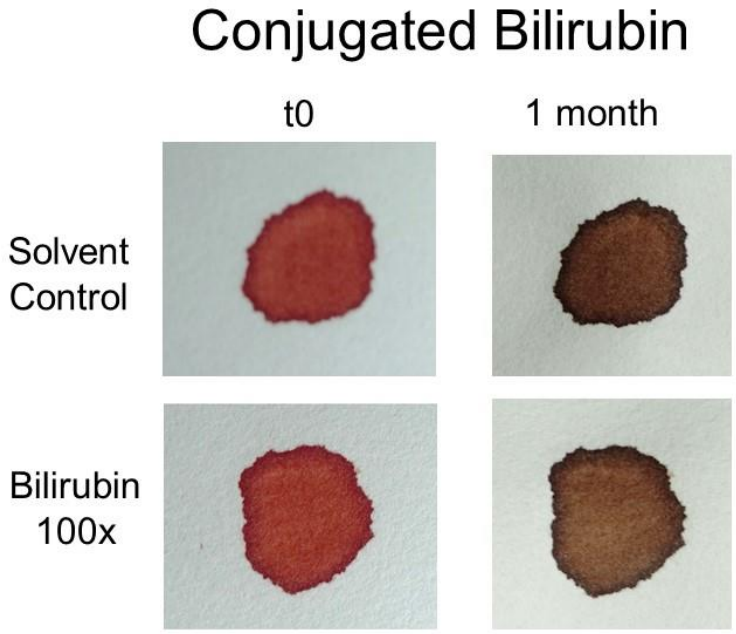


**Figure 10. Effect of high bilirubin (conjugated) on bloodstain color.** Whole blood was mixed with conjugated bilirubin corresponding 100x normal levels (see Materials and Methods for details), then transferred to filter paper. Color was evaluated immediately and 1 month later. The data shown are representative of three separate experiments.

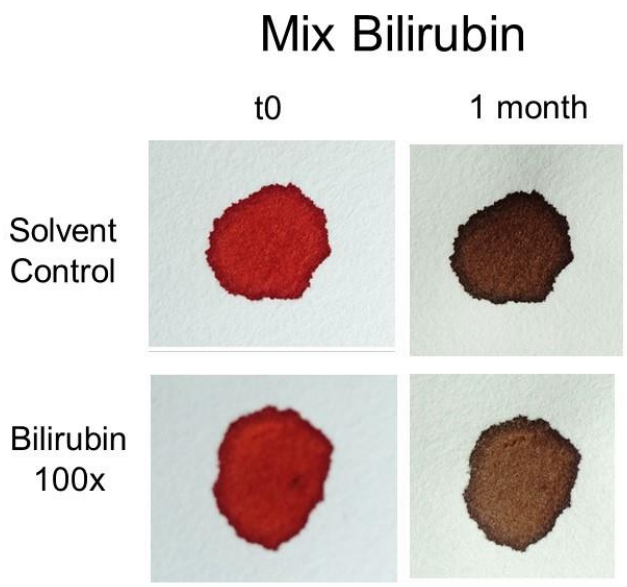


**Figure 11. Effect of high bilirubin (unconjugated + conjugated) on bloodstain color.**

Whole blood was mixed with unconjugated and conjugated bilirubin corresponding 100x normal levels (see Materials and Methods for details), then transferred to filter paper. Color was evaluated immediately and 1 month later. The data shown are representative of three separate experiments.

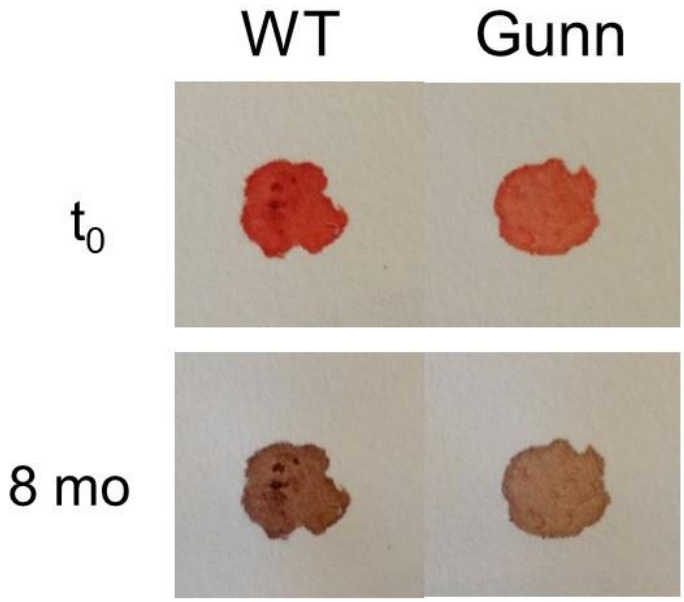


**Figure 12. Reddish color does not persist in blood from Gunn rats, a rodent model containing high levels of endogenous bilirubin *in vivo*.** Blood from Gunn rats was transferred to filter paper, and color was evaluated immediately and 8 months later. The data shown are representative of three separate experiments.

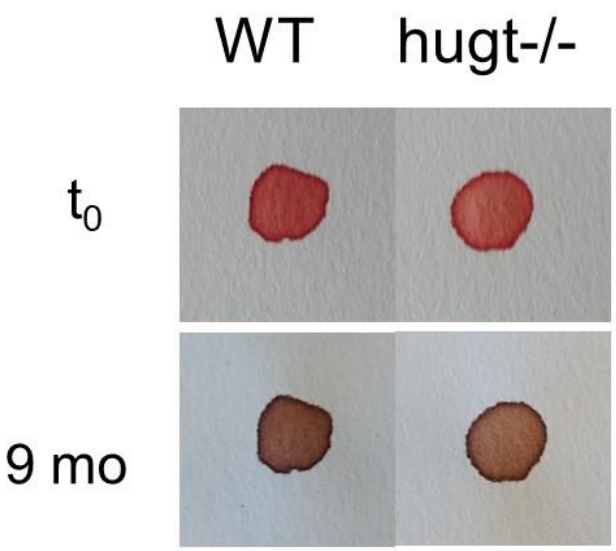


**Figure 13. Reddish color does not persist in blood from hugt -/- mice, a rodent model containing high levels of endogenous bilirubin *in vivo*.** Blood from wild type (WT) or hugt -/- mice was transferred to filter paper, and color was evaluated immediately and 9 months later. The data shown are representative of three separate experiments