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The influence of coagulation on the drying dynamics of blood pools

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The influence of coagulation on the drying dynamics of blood pools

Abstract

Little is currently known about the importance of clotting during the drying of blood pools. While this is of little moment for droplets where drying occurs faster than contact-phase-induced clotting, clotting may significantly influence blood pools drying as it transform a liquid into a gel. To investigate this influence, we compare the drying of citrated and unmodified blood pools at constant haematocrit, showing large morphological differences during drying, both in the surface appearance, in the colour lightness, as well as in the generation and location of cracks. Further, we designed a clotting-reactivation protocol which allowed recovering the morphological evolution of pure blood drying while using citrate-sampled blood. This result opens the way to the use of citrated blood in blood pools investigations.

Keywords: BPA, blood pools, drying, evaporation, fluid dynamics, coagulation

1. Introduction

A very common type of bloodstain found on the crime scene following a deadly blood shedding event, is the blood pool, created by the accumulation of blood on a surface (James 2005). Currently, information that can be obtained from an undisturbed blood pool is very limited, e.g. the volume of a blood pool and if it could constitute loss of life (Bartz 2003), or for other crime scene reconstruction purposes (Lee 1986, Santet 2012, Laan 2014, Laan 2015). Up to date, in most bloodstain pattern analysis studies, blood is used with anti-coagulant. In the case of small drops and trajectories reconstruction, this is not an issue as drying occurs faster than the contact phase clotting. Concerning blood pools however, two different studies have recently investigated their morphology while drying, for diluted pools (Ramsthaleret 2016) and whole blood pools (Laan 2016) for the purpose of estimating the creation time of the blood pools. Both of these studies have suggested that clotting of blood may be an important phenomenon and *a priori* indicate that only freshly drawn whole blood should be used. However, a direct comparison of the drying of blood pools with and without clotting is lacking. Furthermore, using on-the-spot drawn blood poses large practical problems such as the sampling base (because of the unavoidable biological variability), as well as the difficulty to investigate large volume blood pools (> 50ml) where donor sampling is practically impossible and ethically questionable. A possible way to overcome those difficulties could be to reactivate clotting in citrated blood obtained from blood banks by using appropriate methods such as those currently used in haemostasis global tests (e.g. Hemker et al 2001, Dassi et al. 2019).

In this paper we aim at evaluating the importance of clotting in the drying of blood pools as well as the potential pertinence of reactivating clotting in blood samples with respect to blood pools drying. To those ends, we compare the drying of blood pools made from pure, citrated and

citrated+reactivated blood. After detailing the blood sampling, clotting reactivation, blood deposition protocols, experimental setup and analysis methods, the drying morphology and kinetics of the different pools are compared to one another. Our results demonstrate that clotting does have an important effect on the morphology of the drying blood pools and that the carefully designed reactivating process we propose produces drying kinetics and morphologies that are very similar to that of unperturbed whole blood.

2. Materials and methods

Experimental setup

To follow in a reproducible way the drying of a blood pool, the environment must be as constant as possible. Therefore each blood pool was created in a glovebox (Jacomex T-Box, V=700L) where the humidity and temperature were kept constant at 35% and 22°C. The humidity and temperature were recorded during drying by means of a hygro-thermometer (Teslo AG, 175-H2, Datalogger, Germany). Within the glovebox, a camera (Nikon D200, resolution: 2592x3872 pixels or Nikon D300s resolution: 2848x4288 pixels both with a 60mm 1:2.8 lens) was suspended directly above the blood pool with appropriate, constant lighting (see fig. 1). The camera was programmed to take one picture every two minutes, with a resolution of roughly 26 to 30 pixels per millimetre.

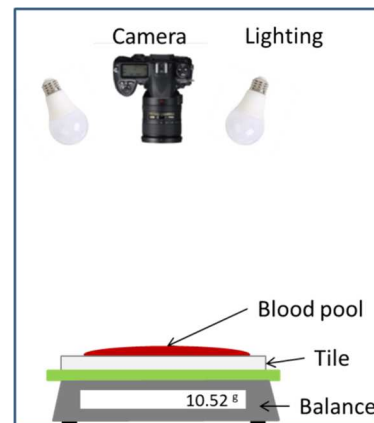


Figure 1 Schematic of the experimental setup.

The substrate for the pools deposition were flat tiles (ref:70728700, Leroy Merlin, France), all tiles being from the same carton. Prior to deposition, the tile surfaces was partially covered with a circular object (petri dish), and subsequently treated with a hydrophobic coating (Ultra Ever Dry, France). Accordingly, the tile had an inner circular hydrophilic part and an outer hydrophobic part, enabling us to reproduce precisely the size and shape of the blood pools, thereby minimizing any influence of the shape of the pool on its drying.

The substrate was sitting on the top of a recording balance (ML802, Mettler Toledo, Switzerland) to determine the mass (m) of the blood pool during the entire drying process (one weight measurement every minute). By means of a reference length positioned next to the pool, several morphological parameters such as the area of the pool, the wet and dry parts of the pool were determined with the help of a Matlab program (see below).

Blood collection

Three different kinds of blood pools were made from the same donor and sampling, namely with: pure blood (no additives), blood containing only the anti-coagulant citrate (no coagulation), and calcium-reactivated clotting blood.

The blood collection procedure was the following.

1. 3 ml of blood were collected in an EDTA tube to make haematocrit measurements. It is very important to start blood drawing with this procedure in order to obtain repeatable results. Indeed the first few ml of blood obtained by venepuncture always contain widely varying amounts of tissue factor, amount which would then completely control the subsequent contact-pathway-induced clotting.
2. 10 ml of blood was collected directly into a 15ml tube (Falcon, Corning, USA), procedure which took up to 2 minutes. This blood was immediately deposited upon the target surface.
3. To make the other two kinds of blood pools, blood was collected in several 3 ml citrated vacuum collection tubes (VenoSafe, Terumo, France) immediately following the collection in the Falcon tube. These tubes contain 10% citrate of the total volume. Since this citrated blood is diluted with respect to whole blood, it effectively decreases the haematocrit value as well as the concentration in proteins. Because red blood cells make from 35% to 50% of the total mass of blood while proteins represent about 10 times less, we chose to correct the haematocrit variation with the procedure described below. One may also note that fibrinogen -the central protein of blood clotting- has a normal variation range of 2 to 4g/L which is therefore impervious to such a small dilution.

Blood reactivation

To reactivate clotting, we use a modified version of the reactivating buffer used in thrombinography or fibrinography (Hemker et al. 2001, Dassi 2019). This buffer is used to reactivate clotting in plasma (1/3 buffer and 2/3 plasma dilution) and therefore contains both tissue factor to activate the extrinsic coagulation cascade as well as phospholipids which mimic the platelet surfaces. In blood pools generated from large wounds, the contact pathway is the most likely clotting activity as the amount of released tissue factor is small compared to the in-vivo vascular breach situation which activates the tissue factor pathway. Therefore, tissue factor was not used in the reactivating buffer proposed here, meaning that eventual clotting will originate from the intrinsic (contact) pathway. Phospholipids were also not used as the present experiments are performed with whole blood which contains platelets. So the essential ingredient to activate the contact pathway is the addition of calcium at an appropriate concentration, also called sometime "recalcification buffer" (Greco 2004).

While important, clotting is not the only process influencing the drying of a blood pools. So, we devised a protocol with the smallest possible dilution while using a reactivating buffer with physico-chemical properties as close as possible to normal blood. Therefore, we used a HEPES buffer with the same osmolarity (300mOsm) and pH (7.4) as normal blood, and added 30g/l of albumin to get approximately the same protein-induced oncotic pressure as normal blood. The obtained dilution ratio is 1/10 buffer 9/10 pure blood, and the buffer composition is 10mM HEPES, 110mM CaCl₂, 30g/L albumin.

The complete protocol is as follows:

1. Collection of 3ml of pure blood in an EDTA tube.
2. Collection in a 15 ml falcon tube. Blood is deposited immediately after collection.
3. Collection in 3ml citrate tubes (i.e. containing 10% in volume of citrate). Tubes are gently mixed by inverting 4 times.
4. Blood is distributed equally over two separate centrifugation tubes which will contain at the end of the procedure:

- a. tube 1, blood + citrate + reactivating buffer
 - b. tube 2, blood + citrate
5. Both tubes are centrifuged at 2500G for 15 minutes.
 6. 20% of the supernatant fluid (plasma) is removed for tube 1 and 10% of the supernatant fluid for tube 2. This ensures the same haematocrit for each sample type after buffer addition.
 7. Platelets are thoroughly dispersed via pipette flushing.
 8. 10% (in initial volume) of reactivating buffer is added to tube 1 and mixed gently by inverting 4 times.
 9. Immediately after the buffer addition and mixing, blood is deposited on the substrate.

This procedure ensures that the final haematocrit, pH, ionic strength and protein content are practically identical for all blood pools made from the same blood sample.

Automated picture analysis

Several image analyses were performed on each photograph of each time series. First, let us recall that each color image acquired by the camera is actually a composite image with three channels (red, green and blue) coded on 8 bits (256 levels). Two kinds of analyses were performed.

First, using a Matlab program, the red channel was extracted from the original jpeg images and the red intensity level for each image was averaged over a 0.5cm^2 disk area close to the center of the pool, where desiccation appears last. This is used to analyse the first stage of drying.

Second, on each RGB image, the Matlab program determined the outer rim, the drying front and the respective dry and wet areas as a function of time as shown in the example displayed in Figure 2. There, the blue line represents the total area of the stain, which reduces slightly in time as the desiccating pool contracts or delaminates. The green line is the separation between the wet area and dry areas and starts to diminish as soon as the drying front is formed.

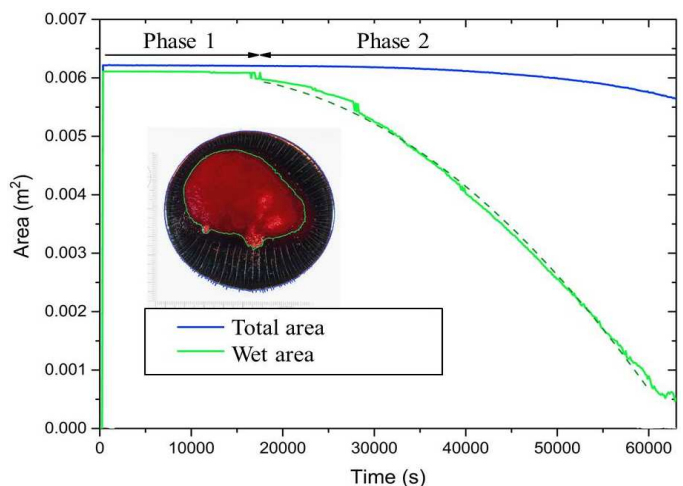


Figure 2. Example of a front analysis. The green line on the pool image is the detected desiccating front separating the dry and wet parts of the pool. The graph shows the temporal evolution of the wet and dry areas as a function of time. The appearance of a dry zone corresponds to the end of phase one. The dashed line is a guide to the eye (power-law fit).

3. Results

Visual drying history of blood pools

The first goal of the present study is to evaluate the effect of coagulation on blood pool drying. To this end, we first compare the visual aspect of pure (clotting) and citrated (non clotting) blood pools obtained from the same donor at the same time, with similar volumes deposited on tiles

and under identical environmental conditions (Cf fig. 3 and its enlargement shown in supplementary file S1). The visual drying history of those blood pools can be separated into two main stages, a simplification of the description published earlier (Laan 2016). Based on the existence of a drying front, we distinguish two phases during drying: the first phase when the drying front has not yet appeared and where the wet area is essentially constant (see fig. 3, left), and the second phase where the drying front propagates from the outer rim to the centre of the stain. The transition between those two phases (i.e. the onset time of the drying front) is in the present case of about 4 hours (see fig. 2). The major feature of the first stage, common to all pools (fig.2), is a large change in colour from dark red to a lighter red. The second stage starts when the rim turns black indicating that the rim starts desiccating. This transition from the red to black colour is referred as the drying front which then propagates towards the centre of the stain until the drying front reaches the centre of the pool. At this point, the pool has almost completely desiccated and is black in colour. The remains contract and the cracks reach the middle of the stain. Accordingly, flakes can separate and partially or completely detach from the surface.

Those two stages are observed for every pool while the precise duration of the stages can differ between blood pools, depending on the donor, humidity, temperature, shape and size of the pool and the kind of substrate.

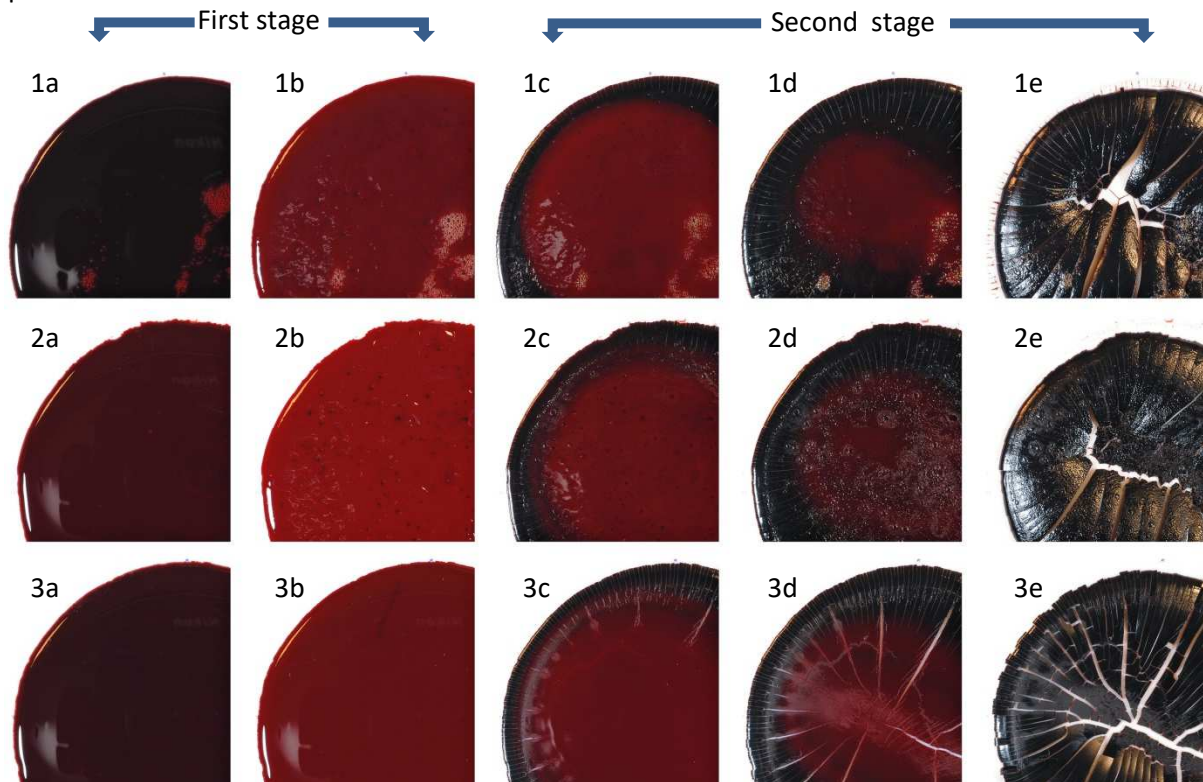


Figure 3: Evolution of drying blood pools at typical times. To better discern details, about 1/4th of the complete circular blood pool is shown. 1a-1e: pure blood; 2a-2e: reactivated blood; 3a-3e: citrated blood. A full resolution of this image is available in the supplementary materials.

First stage: sedimentation and clotting.

During stage I, the surface of pure blood (images 1a to 1e in Fig. 3) transits from a dark glossy surface (image 1a) to a clearer (image 1b), matte, irregular and solid-like surface suggesting the occurrence of clotting. A relatively large number of small black dots is also observed in figure 1b, probably consisting in small dense clots. The evolution of the reactivated blood shows the same

features. In contrast, the non-coagulating citrated blood stays glossy with a smooth surface (image 3b of fig.3) strongly suggesting that citrated blood remains liquid throughout the first stage.

A striking feature of the first stage –on top of the visual aspect change- is the change in colour. To show this evolution, we plot in fig.4 the red channel intensity of the red-green-blue (RGB) images acquired by the camera (see materials and methods). The curves show a clear difference between clotting and non-clotting blood. Indeed, while all bloods show a similar increase during the first hour, both clotting bloods show a sharp increase in red channel intensity after about 1 hour while the red channel increase is monotonous for non-clotting blood. Since the sudden increase is only found in pools where clotting is active, it should be the consequence of clotting.

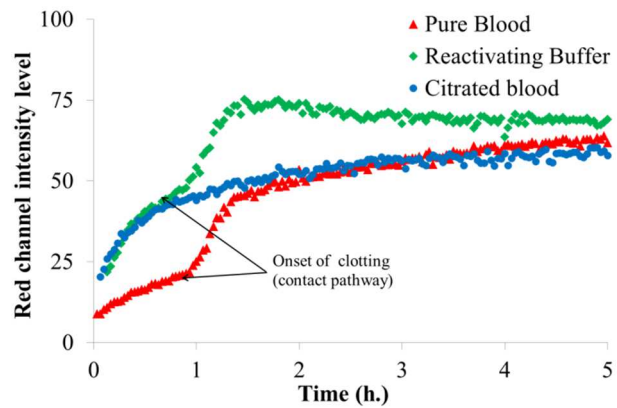


Figure 4. Evolution of the red channel vs time. The red channel was averaged over a small area in the center of each photograph

Second stage: drying and desiccation

Visually, the second stage in figure 2 is roughly similar in all three bloods, with the black rim growing in width with time. However, an important visual difference between the clotting and non-clotting pools can be observed. For pure and reactivated blood (fig2 images 1.c to 1.e and 2c. to 2e.), cracks are formed only *within* the desiccating rim, but do not transgress past the desiccating front boundary. In contrast, for citrated blood (fig1 images 2.c to 2.e), the cracks propagate much further than the desiccated region.

To investigate in more detail the similarities and differences we turn now to a quantitative analysis of the data. In Fig. 5 the mass and evaporation rate for the different blood pools is plotted as a function of time. The oscillations in the evaporation rate are caused by the humidity chamber turning on and off to keep the humidity within a certain range. The central observation is that both the mass and evaporation rate for all the pools are very similar. This somewhat surprising result is similar to the one obtained previously (Laan 2016 fig. 6) when using pure blood from several donors and exploring drying for different humidity.

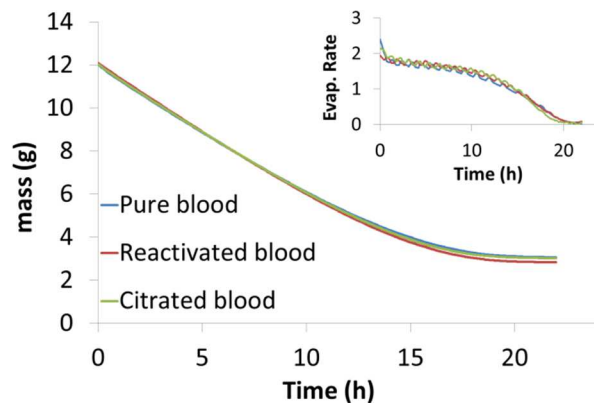


Figure 5 Temporal evolution of the mass of the blood

The most interesting and sensitive quantity is actually the dry area evolution, which is simply the difference between the total area and the wet area as a function of time. As seen in fig. 6, both clotting bloods dry in a similar manner, following approximately a power law, while the non-clotting blood dries in a much more complex way.

4. Discussion

Currently, most bloodstain pattern analysis studies use blood with anti-coagulant which, in the case of small droplets is not a problem as drying finishes prior to the occurrence of contact-pathway-induced clotting. However, when larger pools are studied, which may take several hours to days to dry, it looks like coagulation has a significant effect on drying, both prior to and during desiccation. Indeed, in the first phase of the drying, when the drying front has not yet appeared, the main morphological parameters of the pool are its textural appearance and color.

First, an initial increase in red channel intensity for all bloods was observed. This phenomenon may be related to the sedimentation of the red blood cells. Indeed, this phenomenon produces a layer with less RBCs close to the top of the pool, layer which absorbs less light and deepens with time. This allows more and more light to come back to the camera since RBCs both scatter and absorb light (Baravian 2007). An alternative explanation maybe found in the large difference of light absorbance of haemoglobin

and oxy-haemoglobin in the red part of the spectrum (~600nm). Indeed since blood is drawn from a vein it has a low oxygen concentration and therefore appears dark, while processing and mixing should increase the oxygen content of citrated bloods. This could explain the difference in appearance of the blood deposited directly after being drawn and the other two which are undergoing centrifugation and mixing. Then, during the first phase of drying, all bloods are in contact with oxygen which may increase the amount of oxy-hemoglobin, and hence lighten its colour.

Then there is a sharp increase in lightness at around 1h for the clotting pools. Such a well-defined increase could provide a new forensic temporal marker which could nonetheless depend on external parameters, in particular the temperature, and perhaps on the pool volume and geometry. This increase is synchronous with the appearance of corrugations on the surface of the pool (images 1b and 2b in fig. 3), showing that at this time the surface (at least) has become solid. Since this phenomenon does not occur in the non-clotting blood pool, this strongly suggests a liquid-gel transition, i.e. the occurrence of clotting. Furthermore, since the blood pool is illuminated from above and recording is also performed from above (see fig. 1), our experiments are somewhat similar to the reflectance measurements of blood clotting performed in quartz cuvettes (Greco 2004, Greco 2007) where a similar sharp increase in reflectance was observed when clotting occurs, albeit for much shorter times. This much shorter time probably comes from the fact that in our experiments there is a blood-air interface which is a much worse contact-pathway clotting promotor than quartz. While the increase in reflectance was clearly associated to blood clotting in Greco's work, the precise origin of this increase remained unclear. Recently however it has been shown that a dense and thin fibrin film is produced at the water-air interface of clot forming in contact with air (Macrae 2018). So, since fibrin appears as a white substance, this sharp increase in whiteness may be the consequence of fibrin formation inside the drying pool and/or at its blood-air interface.

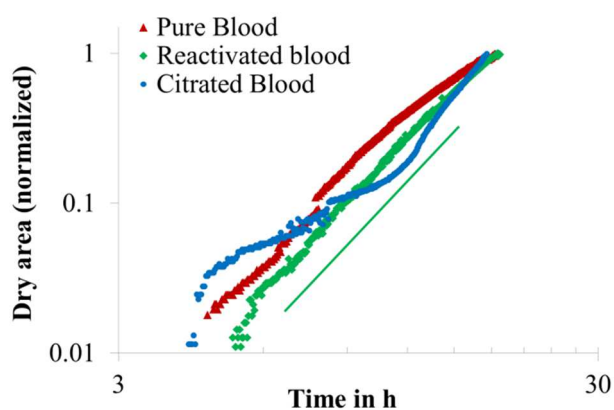


Figure 6 Evolution of the dry area versus time for the three bloods.

Surprisingly, very little differences were found in the temporal evolution of the mass of the pools, observation which remains to be explained. Conversely, the effect of clotting is morphologically evident during the second part of the drying since cracks propagated to the wet part of the pool in the non-clotting case while they did not for clotting bloods. Furthermore, the dynamics of the advance of the drying front was also significantly different according to the clottability of the bloods, the clotting bloods showing simpler, almost power law dynamics.

Finally, it appears that the citrated blood reactivation protocol proposed in the present article provides results that are in fair agreement with those obtained from the pure sample. This result opens the way to the use of reactivated citrated blood in bloodstain pattern analyses.

4. Conclusion

In this study we investigated whether clotting-reactivated blood dries in a similar fashion to pure blood. From our results it is clear that if blood does not coagulate, the drying mechanics are significantly different and the final appearance of the stain is also significantly different from that of pure blood. Furthermore, non-coagulating blood shows that the speed of the drying front is strongly non-monotonous in contrast to pure blood. Fortunately, the clotting of blood containing citrate can be reactivated by using a carefully designed reactivating buffer. By doing so, clotting-reactivated blood dries almost identically to pure blood with both the visual appearance of the pool and the results of the analysis of the drying front being very similar. Accordingly, reactivated blood may be used for experiments requiring blood for the most realistic situations, where it is not possible to obtain pure blood, e.g., when large volumes of blood are required.

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