Journal of Diabetes Science and Technology
Volume 5, Issue 3, May 2011

SYMPOSIUM

Metabolic Biofouling of Glucose Sensors *in Vivo*: Role of Tissue Microhemorrhages

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Abstract

Objective:

Based on our *in vitro* study that demonstrated the adverse effects of blood clots on glucose sensor function, we hypothesized that *in vivo* local tissue hemorrhages, induced as a consequence of sensor implantation or sensor movement post-implantation, are responsible for unreliable readings or an unexplained loss of functionality shortly after implantation.

Research Design and Methods:

To investigate this issue, we utilized real-time continuous monitoring of blood glucose levels in a mouse model. Direct injection of blood at the tissue site of sensor implantation was utilized to mimic sensor-induced local tissue hemorrhages.

Results:

It was found that blood injections, proximal to the sensor, consistently caused lowered sensor glucose readings, designated temporary signal reduction, *in vivo* in our mouse model, while injections of plasma or saline did not have this effect.

Conclusion:

These results support our hypothesis that tissue hemorrhage and resulting blood clots near the sensor can result in lowered local blood glucose concentrations due to metabolism of glucose by the clot. The lowered local blood glucose concentration led to low glucose readings from the still functioning sensor that did not reflect the systemic glucose level.

J Diabetes Sci Technol 2011;5(3):583-595

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Abbreviations: (AAH) acute artificial hemorrhage, (CGM) continuous glucose monitoring, (DAH) delayed artificial hemorrhage, (DPI) days post-implantation, (GSF) glucose sensor function, (HWB) heparinized whole blood, (RBC) red blood cell, (TSR) temporary signal reduction, (WB) whole blood

Keywords: artificial hemorrhages, blood clots, continuous glucose monitoring, implantable glucose sensor, metabolic biofouling, mouse, sensor function *in vivo*, temporary signal reduction, tissue hemorrhages

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Introduction

Despite the fact that implantable glucose sensors have existed since the 1980s, our knowledge of the various factors and tissue responses that compromise both short- and long-term glucose sensor function (GSF) *in vivo* is still limited. It is thought that sensor-induced inflammation and fibrosis tends to result in gradual or even complete loss of sensor function at later stages (weeks to months post-implantation)[1,2] *in vivo*, but other types of unexplained loss of sensor function have been observed. For example, commercial subcutaneous glucose sensors (having 3–7 day lifetimes) are generally less accurate in the 24-hour period immediately post-implantation than in the subsequent days of the implantation period. This relatively reduced accuracy is caused, in part, by periods of lowered sensitivity, where sensitivity is defined as the ratio of the sensor glucose reading to a reference value. This lowered sensitivity, which does not occur in all implants, can last for several hours and often resolves within 24 hours post-implantation.

We have demonstrated that a rapid loss of sensor signal was associated with blood clot formation when glucose sensors were incubated with whole human blood at 37 °C *in vitro*.[3] This rapid sensor signal loss was not seen when glucose sensors were incubated with heparinized whole blood (HWB) or other blood components.[3] Additionally, we demonstrated that blood clot interference with sensor function *in vitro* was not related to (1) proteins, including clot proteins, (2) plasma-derived clots, or (3) leukocytes. It was also found that blood-based interference with GSF *in vitro* correlated with the increase of red blood cell (RBC) density around the sensor. Based on these results, we concluded that the accumulation of high levels of RBCs around the glucose sensor resulted in a dramatic fall in local glucose levels as a result of RBC metabolism within the local sensor microenvironment. This local RBC glucose consumption at the site of sensor location ultimately resulted in the fall in sensor output. This RBC-based "metabolic sink" created the appearance of loss of sensor function, but in reality, the sensor was reporting the true (lowered) glucose levels within the microenvironment surrounding it.

In the present work, we extend the *in vitro* study to our mouse model with continuous glucose monitoring (CGM) to evaluate the effect of "artificially" induced hemorrhages (i.e., subcutaneous injections of blood) on GSF *in vivo*.

Research Design and Methods

Effect of Whole Blood on Glucose Sensor Function in Vitro: Abbott Diabetes Care Navigator and DexCom Seven Sensors

The *in vitro* impact of human blood on glucose sensor performance was evaluated using commercial glucose sensors, i.e., Navigator™ (Abbott Diabetes Care, Alameda, CA) and Seven® (DexCom™, San Diego, CA). These *in vitro* studies were conducted for up to 24 h using our *in vitro* sensor function chambers and CGM system as previously described.[3] Heparinized whole blood and non-HWB was obtained from healthy nondiabetic individuals and injected directly into the container holding the sensor. Sensor leads for the Navigator and Seven sensors were connected to a potentiostat and data acquisition system. Potentials of +40 and +600 mV were applied to the working electrodes of the Navigator sensor and the Seven sensor, respectively. Once blood was added to the sensors, the CGM system was immediately initiated.

Mice, Sensor Implantation, and Continuous Glucose Monitoring

Female ICR (CD-1) mice ranging from 21–50 g in weight were utilized for these *in vivo* studies and were obtained from Harlan (Indianapolis, IN). Navigator glucose sensors utilized in these *in vivo* studies were obtained from Abbott Diabetes Care, and Seven glucose sensors were obtained from DexCom, Inc. Glucose sensors were implanted into mice, and CGM was followed as described with modifications.[4,5] For sensor implantation, a small volume of saline (50–100 μl) was injected subcutaneously at the location for sensor implantation utilizing a 26 ga needle. Next, using a 23 ga needle, a small opening was made at the saline site, and the sensor tip was carefully inserted. Using this procedure allowed the sensor tip to be visible through the skin of the mouse, which was important for later blood injections (**Figures 1A** and **1B**).[4] Implanted sensors were secured to the mouse skin, and CGM was initiated as previously described.[4–6] Blood was periodically sampled from a tail snip to obtain reference glucose levels using a FreeStyle glucose meter (Abbott Diabetes Care, Alameda, CA). Sensor output data were retrospectively calibrated using average sensitivities calculated from all non-temporary signal reduction (TSR) data pairs. Laboratory Animal Care Use Committee at the University of Connecticut, Farmington, approved all mouse studies.

Mouse Blood Collection

Mouse blood was obtained from the saphenous vein using the protocol described by Hem and colleagues[7] with modifications. Briefly, the hind leg of the mouse was shaved the day before the experiment was conducted using a hair remover lotion (e.g., Nair). The next day, a thin layer of Vaseline was added to the shaved hind leg of the anesthetized mouse to prevent blood clotting on the hair. Next, a 23 ga needle was used to puncture the saphenous vein, and the blood was collected into the Microvette CB 300 (SARSTEDT, Nümbrecht, Germany). Up to 100 µl of mouse blood can be collected utilizing this method. It should be noted that it is important to take the blood from a major vein, such as the saphenous vein, to assure rapid and adequate blood collection prior to blood clotting. In addition, it is important to keep all tubes and syringes utilized for blood collection on ice prior to use in order to minimize blood clotting in the syringe or tubes. Blood injection at site of sensor implantation occurred immediately following blood collection (*vide infra*).

In Vivo Models of Hemorrhage and Blood Clots

The general approach for these studies was to obtain mouse blood from the saphenous vein of the mouse to inject the mouse blood directly at the site of sensor implantation. Blood injections were done immediately [acute artificial hemorrhage (AAH) model] or 24 h post-sensor implantation [delayed artificial hemorrhage (DAH) model] to produce an artificial hemorrhage at the site of sensor implantation.

Acute Artificial Hemorrhage Model

To simulate acute tissue trauma with hemorrhage that can result from sensor insertion, we developed an AAH model. For this model, on the day of sensor implantation, the skin of an ICR mouse was disinfected using 70% isopropyl alcohol and a sensor was implanted as described earlier. In order to ensure that sensor displacement was unlikely during the time of blood clotting, the sensor was secured to the mouse skin prior to blood injection as previously described[4,5] (**Figure 1C**). Blood was collected shortly after implantation from the saphenous vein from the anesthetized mouse. Immediately following blood collection, 50 µl of blood was injected at site of sensor implantation. Insulin syringes were utilized for blood injections in order to minimize the amount of blood needed for the injection.

Delayed Artificial Hemorrhage Model

To simulate delayed tissue trauma with hemorrhage resulting from sensor movement, we developed a DAH model. For this model, we implanted sensors into a saline bleb utilizing the implantation approach described earlier (**Figure 1A and 1B**). Sensor output was followed continuously for approximately 24 h prior to blood injection at the site of sensor location. Although our blood *in vitro* study demonstrated that sensor functionality drop only occurred in the presence of whole blood (WB) but not HWB,[3] we tested sensor response after injection of HWB or WB at the site of sensor implantation. For these studies, mice were allowed to roam freely in their cages post-sensor implantation, as well as post-WB or HWB injections. For the blood injection, mice were anesthetized, the nylon mesh around the sensor tip was removed (**Figure 1C**), and blood was collected and immediately injected at site of sensor implantation (**Figure 1D**). Mice were transferred back into their cages, and the experiment was terminated 24–48 h post-blood injections.

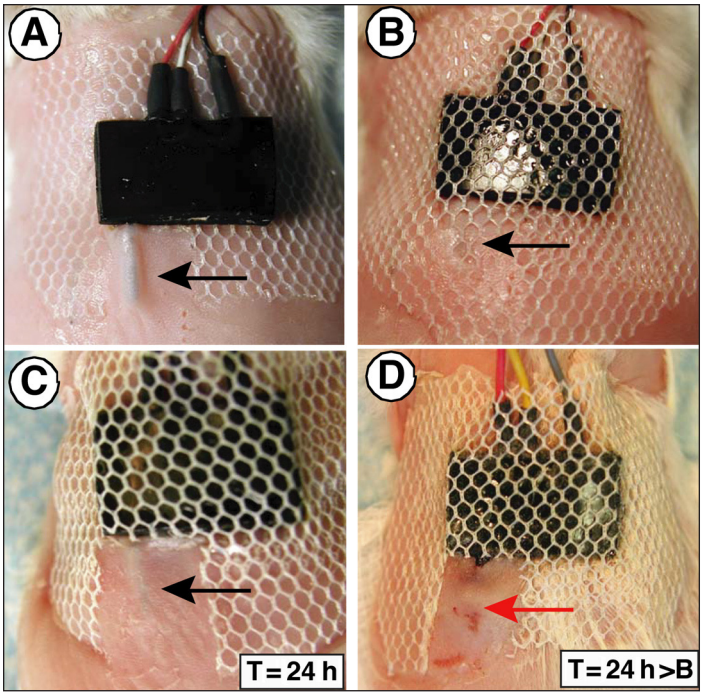

Figure 1. Mouse model of blood-induced TSR: sensor implantation and blood injection. **(A)** CD-1 mouse with implanted glucose sensor and **(B)** protective mesh to secure sensor to skin 0 h post-sensor implantation. **(C)** At 24 h post-sensor implantation, part of protective mesh is removed **(D)** to allow for blood injection at the site of the sensor tip.

Impact of Blood Protein Injections on Glucose Sensor Function in Vivo

To investigate if edema (i.e., liquid component of blood that leaks into the tissue) caused by vascular permeability could alter glucose sensor dynamics, we injected plasma derived from mouse blood directly at the site of sensor implantation. Since plasma contains glucose, we also tested

whether injection of saline, with varying concentrations of glucose, would temporarily or permanently alter sensor output when injected at the site of sensor tip location. For these studies, only Abbott Diabetes Care sensors were utilized, and sensors were implanted in CD-1 mice as previously described by our laboratory.[5] Immediately after sensor implantation, CGM was initiated using the system described by our laboratory.[5] After a 24 h run-in period, 50 µl of either plasma or glucose solution was injected at the site of sensor implantation (i.e., sensor tip) in the mouse.

For the sensor studies described earlier, we used 1 sensor per mouse. In the case of the Abbott Diabetes Care Navigator sensor, we used at least 10 sensors per treatment/injection condition (i.e., saline, plasma, WB, HWB). In the case of the DexCom Seven sensors, we utilized at least 10 sensors for the WB injection treatment and a minimum of 3 sensors for the HWB or saline treatment condition.

In Vivo Sensor Functionality during Temporary Signal Reduction

In order to investigate sensor response and functionality after blood injection and TSR occurrence, we administered mice intraperitoneally a glucose bolus injection. For this study, we injected 1–2 g/kg glucose intraperitoneally into CD-1 mice a few hours after TSR occurrence. Blood glucose reference measurements were obtained from a tail snip every 10 to 20 min.

Histopathologic Analysis of Tissue Reactions at Glucose Sensor Implantation Sites

In order to evaluate tissue responses to glucose sensor implantation at various time points, individual mice were euthanized and the tissue containing the implanted sensors were removed, fixed in formalin buffer for 24 h, followed by standard processing, embedded in paraffin, and sectioned. The resulting 4–6 µm sections were then stained using standard protocols for hematoxylin and eosin. Qualitative histopathologic evaluation of tissue reactions at sites of sensor implantation was performed on mouse specimens obtained at 1, 2, or 3 days post-implantation (DPI) of the glucose sensor. Resulting tissue sections were evaluated directly and documented by digitized imaging using an Olympus Digital Microscope. Histological evaluation included disruption of tissue architecture; evaluation of cell death; edema; inflammation, including leukocyte populations; as well as presence and distribution of RBCs as an indication of hemorrhage (naturally occurring or artificially induced).

Results

In Vitro Effect of Whole Blood and Heparinized Whole Blood on Glucose Sensor Function

To investigate the effect of blood and blood clots on sensor function, we evaluated the *in vitro* impact of HWB and WB on DexCom Seven and Abbott Diabetes Care Navigator glucose sensor functionality, utilizing our previously described *in vitro* model.[3] To achieve this, we incubated sensors in freshly drawn HWB and WB from nondiabetic individuals (starting blood glucose levels approximately 100 mg/dl). As can be seen in **Figure 2,** both DexCom and Navigator sensors displayed a much more rapid fall in output when exposed to WB than when exposed to HWB. It was observed that, in the case of WB, blood clot was quickly formed around both types of sensors upon incubation at 37 °C. In the case of HWB, the RBCs quickly settled to the bottom and the blood separated into plasma and RBC layers with approximately the same volume. The slow decline in sensor response in HWB over the 5 h testing period was due to the decrease in glucose levels in the blood caused by RBC glucose metabolism as found in our previous study.[3] It should be noted that, in both cases, normal sensor function was restored after washing the sensor with phosphate-buffered saline and testing with glucose (data not shown). These results are consistent with our previously published *in vitro* blood sensor data.[3] More importantly, this study indicates that the reduced signal observed in the presence of blood clots is not due to sensor malfunction, but rather to a clot induced local reduction of the glucose concentration.

Effect of Fluid Injections on Sensor Function in Vivo

Because our hemorrhage models are based on the injection of blood at sites of sensor implantation in mouse skin, we first studied the effects of direct injection of other fluids on sensor function *in vivo*. An influx of fluids such as plasma or serum could impact glucose sensing in at least two fashions: (1) sensor biofouling and (2) changes in glucose dynamics. Related to sensor biofouling by plasma or serum, we have previously demonstrated that, *in vitro*, neither serum nor plasma interferes with sensor function over a 48 h time period.[3] Here we extended this *in vitro* study to our mouse model to test the effect of direct fluid injections at the site of sensor implantation.[4–6] The injected fluids included 50 µl of (1) saline, (2) saline + glucose, and (3) mouse plasma at the sensor tip (injection time is indicated by green arrow). **Figures 3A**, **3B**, **3E**, and **3F** show the calibrated continuous glucose sensing data. It can be seen that, when saline without glucose was injected at the sensor tip, a brief fall in sensor

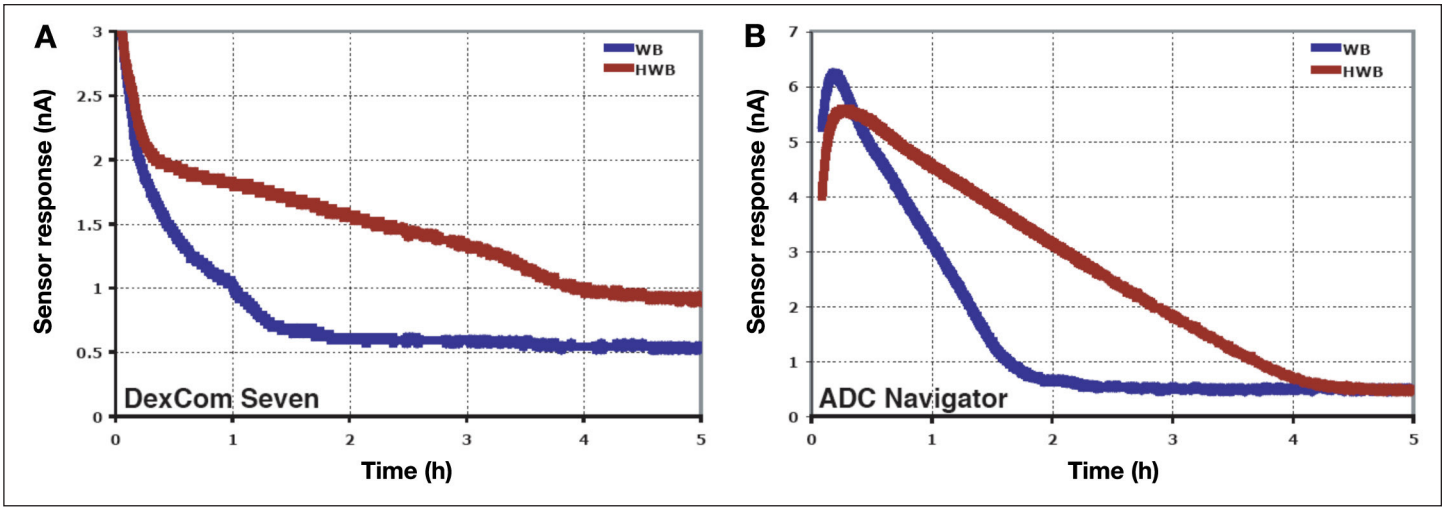

Figure 2. Effect of HWB and WB on GSF *in vitro*. To determine the impact of HWB or WB on sensor function in vitro, approximately 1 ml of HWB or WB was added to the reaction vessel containing a glucose sensor and incubated at 37 °C with continuous glucose sensing. Reaction vessels were sampled for blood glucose levels at the beginning and end of the experiment by utilizing an external glucose monitor. These studies were repeated at least twice per glucose sensor. **(A)** Results of one representative study utilizing the DexCom Seven sensor for HWB and WB. **(B)** Results of one representative study utilizing the Abbott Diabetes Care Navigator sensor for HWB and WB.

output was apparent, most likely as a result of the dilution of the interstitial glucose by saline (**Figures 3A** and **3B**). When saline solution containing glucose (406 mg/dl) was injected at the implanted sensor tip location, it caused a brief increase in sensor output (data not shown). It appears this short increase in sensor output reflects the increase in the local glucose levels at the site of injection. Additionally, histological evaluation of the saline injection at sites of sensor implantation indicated no significant histopathologic effects in the mouse skin (**Figures 3C** and **3D**). Next, when mouse plasma (plasma glucose level of 210 mg/dl) was injected at the implanted sensor tip, it caused a brief spike in blood glucose levels and sensor output (**Figures 3E** and **3F**). When the plasma injection sites were evaluated histologically, we saw no differences when compared with saline-injected sensor implantation sites (**Figures 3G** and **3H**). In summary, these results demonstrated that injected fluids or fluid leakage at the site of sensor location did not induce any extended sensor function loss. The temporary variation in sensor output reflected the local glucose concentration change caused by fluid injection. Assuming that whatever solution is injected at or leaks into the site of sensor implantation has the same glucose level as the mouse, an extensive change in sensor output caused by that fluid (as would be the case for edema) is probably unlikely.

Effect of Artificial Hemorrhages on Sensor Function in Vivo

Acute Artificial Hemorrhage Model

To simulate acute tissue trauma with hemorrhage that can result from sensor insertion, we developed an AAH model. In this model, mouse blood was injected directly at the site of sensor implantation immediately after sensor insertion, and sensor function was then continuously monitored. As expected, saline injections at the sensor implantation site had no significant effects on sensor function in our mouse model (**Figures 4A**, red arrow point). As stated earlier, saline injected at the site of sensor implantation can temporarily cause oscillations of the sensor signal, most likely due to dilution of the interstitial glucose by the saline injection. This drop of sensor output lasts usually approximately 10 to 15 min for an injected saline volume of 50 µl. Alternatively, when mouse blood was injected at the site of sensor implantation, there was a dramatic fall in sensor output (**Figure 4B**, red arrow point). This fall usually lasted several hours before the sensor output resumed to its normal range.

Delayed Artificial Hemorrhage Model

The studies described earlier clearly demonstrated that acute hemorrhages, which can occur at the time of sensor implantation, interfere with GSF *in vivo*. To further explore this phenomenon, we delayed the blood injection until after 24 h of normal sensor operation to evaluate the impact of delayed hemorrhage formation on GSF. We hypothesize that this model mimics what occurs when movement of implanted sensors damages nearby blood vessels and induces hemorrhages, i.e., delayed hemorrhages. Injection of saline in this model for both sensor types (DexCom Seven and Abbott Navigator) caused only a brief decrease in sensor output (**Figures 5A** and **5D**). When WB was injected at the site of sensor implantation,

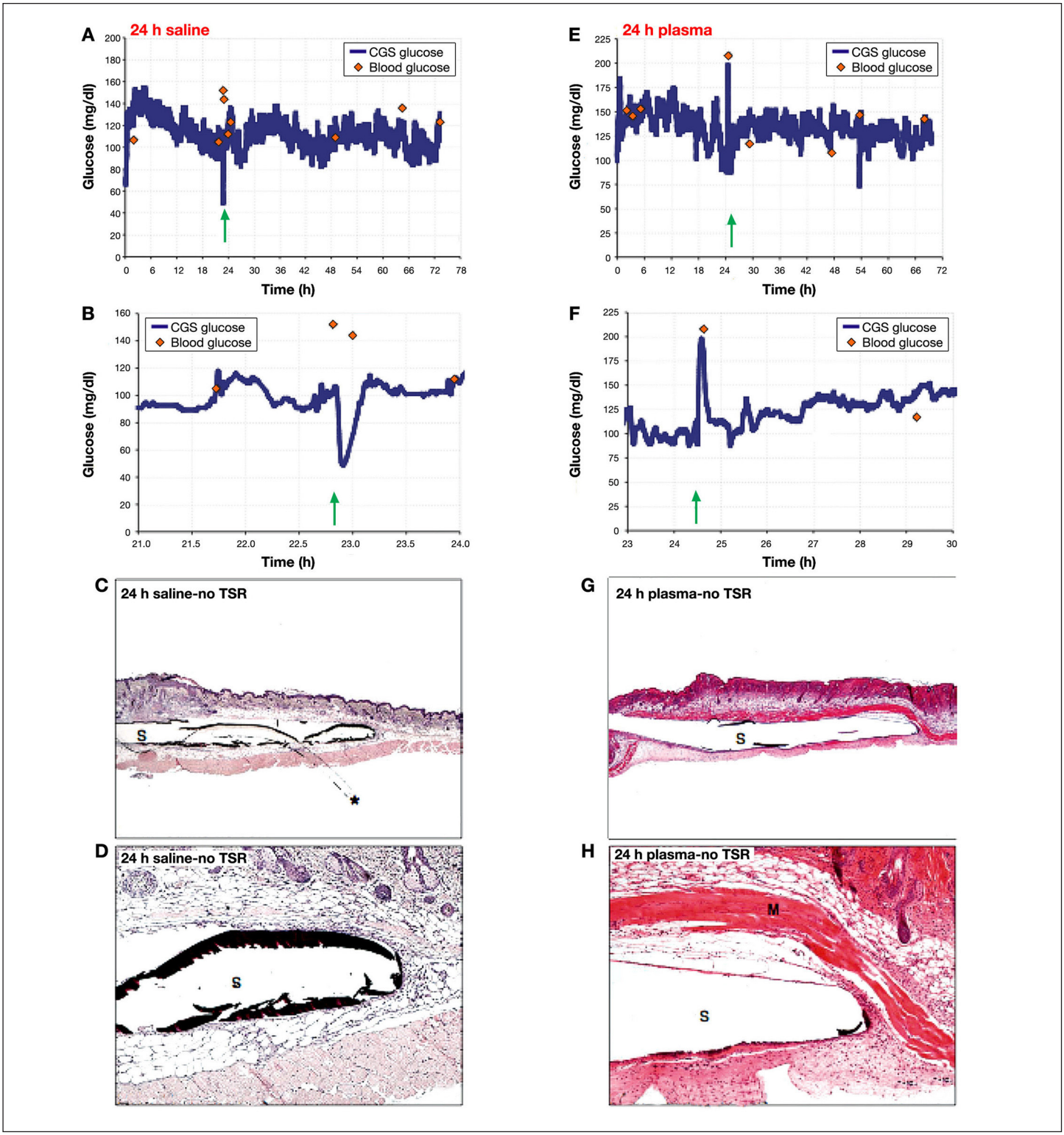

Figure 3. Impact of saline and plasma on continuous glucose sensing in CD-1 mice. Calibrated continuous glucose data for Navigator sensors implanted in CD-1 mice. Representative results are shown for CGM in CD-1 mice with **(A–D)** saline injection and **(E–H)** plasma injection (continuous glucose sensor glucose = solid blue line; blood glucose levels using a FreeStyle glucose monitor = red diamonds; green arrow = time of saline or plasma injection). **(B)** A magnified view of the data from A for the period 21–24 h after implantation, and **(F)** a magnified view of the data from E for the period 23–30 h after implantation. To evaluate tissue reactions at sites of sensor implantation, mouse tissue was obtained from the sensor tissue sites at 3 DPI. The resulting tissue was processed for histopathologic evaluation as described in Materials and Methods. Standard hematoxylin and eosin staining was used for this study. The original sensor location is designated by "S," and residual sensor coating is designated by the asterisk. **(D)** A magnified view of the histopathologic tissue section from **C**. **(H)** A magnified view of the histopathologic tissue section from **G**. CGS, continuous glucose sensor.

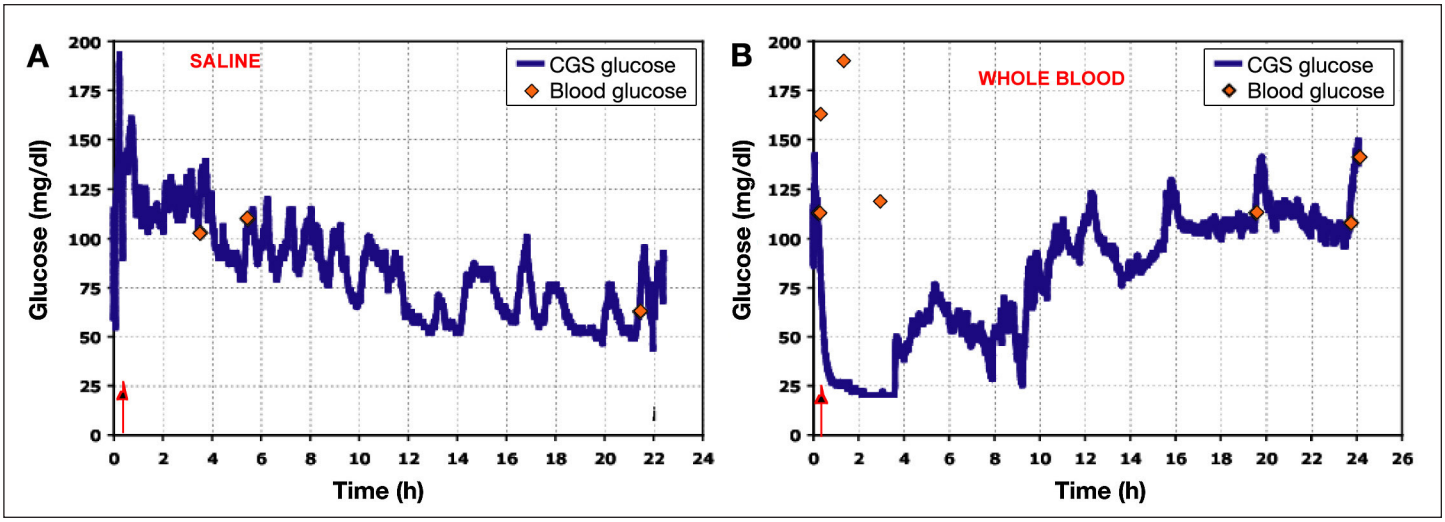

Figure 4. Impact of AAHs on sensor function *in vivo*. Calibrated continuous glucose data for sensors implanted in CD-1 mice. Continuous glucose sensor and blood glucose levels were evaluated for up to 24 h after sensor implantation (continuous glucose sensor glucose = solid blue line; blood glucose levels using a FreeStyle glucose monitor = red diamonds). **(A)** Injection of saline 10 min post-sensor implantation. **(B)** Injection of mouse blood 10 min post-sensor implantation. CGS, continuous glucose sensor.

both sensors' response sharply fell shortly after blood injection (**Figures 5B** and **5E**). The sensor outputs remained at the background level for several hours and then slowly resumed to their normal range. These studies demonstrate that WB/blood clots can interfere with glucose sensor output *in vivo*. As was the case with WB injection, HWB also resulted in a temporary loss of sensor functionality for both the DexCom Seven and the Abbott Diabetes Care Navigator (**Figures 5C** and **5F**). However, in some cases, sensor function declined slowly over several hours compared with a sharper fall in sensor response for WB (**Figure 5F**). This extended period of sensor decline might be due to a delay in blood clotting induced by heparin, which would allow the dissipation of the metabolically active RBCs. It should also be noted that, in these *in vivo* studies, the pressure of the tissue mass itself will press RBCs directly against the sensor and thereby induce a "metabolic barrier" to glucose diffusion.

Correlation of Blood Proximity and Glucose Sensor Function in Vivo

It is likely that the proximity of the blood to the sensing element of the sensor (tip) influences the occurrence and duration of TSR *in vivo*. We hypothesized that any lack of TSR occurrence after blood injection is the result of blood accumulation occurring distant to the sensing element of the sensor. Furthermore, we hypothesized that close proximity of the blood to the sensing element of the sensor is critical to seeing the TSR effects *in vivo*. To test this hypothesis, we correlated sensor function (TSR versus non-TSR) with the proximity of the injected blood to the sensing element of the glucose sensor. For this study, sensor function was evaluated post-blood injection as occurrence of TSR versus no TSR. Next, the tissue around the sensor was harvested and evaluated for blood distribution by histopathology. When sensor functionality was correlated with blood location in tissue sections where no TSR occurred, we found that blood accumulation was close to the sensor but not in close proximity of the sensing element (**Figures 6A**, **6B**, and **6C**). This lack of TSR occurrence (**Figures 6B** and **6C**) was likely due to the fact that sensor and RBCs are separated by a layer of fat and/or muscle cells, thus preventing an RBC-induced "metabolic barrier" at the sensor interface from being created. Alternatively, TSR was associated with intimate/direct contact of the blood with the sensors *in vivo* by gross and histopathologic analysis (**Figures 6D, 6E**, and **6F**).

Blood Creates a "Metabolic Sink" for Glucose at Sensor Implantation Sites

In order to investigate the mechanism(s) related to sensor performance during TSR occurrence, an intraperitoneal glucose bolus injection (80 µl of a 0.5 g/ml injectable dextrose solution) was administered approximately 5 h post-blood injection (**Figures 7A** and **7B**). Despite an acute loss of sensor function, an elevation of sensor response was observed post-glucose bolus injection. We attributed this temporary delayed rise in sensor response to the delivery of a high glucose supply. With this fast glucose delivery, it is impossible for the RBCs at site of sensor location to metabolize the high supply of glucose, which, in turn, allows the glucose to diffuse to the implanted sensor, hence increasing

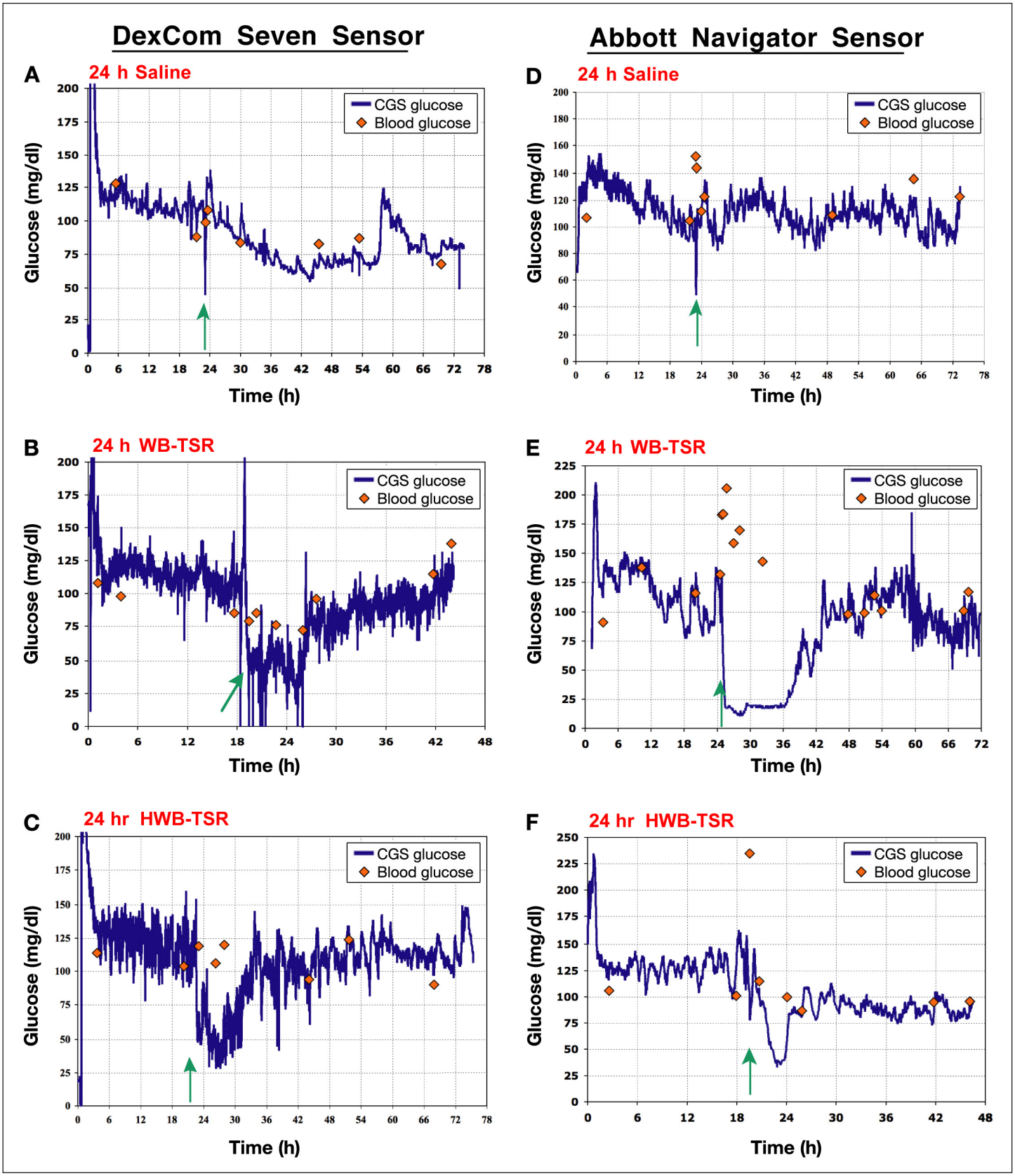

Figure 5. Impact of saline and blood on continuous glucose sensing in CD-1 mice. **(A–C)** Calibrated continuous glucose data for DexCom Seven sensors. **(D–F)** Calibrated continuous glucose data for Abbott Diabetes Care Navigator sensors. Continuous glucose sensor and blood glucose levels were evaluated for up to 75 h after sensor implantation (continuous glucose sensor glucose = solid blue line; blood glucose levels using a FreeStyle glucose monitor = red diamonds; green arrow indicates time of saline or blood injection). **(A, D)** Saline injection 24 h post-Seven or Navigator sensor implantation. **(B, E)** WB injection 24 h post-Seven or Navigator sensor implantation. **(C, F)** HWB injection 24 h post-Seven or Navigator sensor implantation. CGS, continuous glucose sensor.

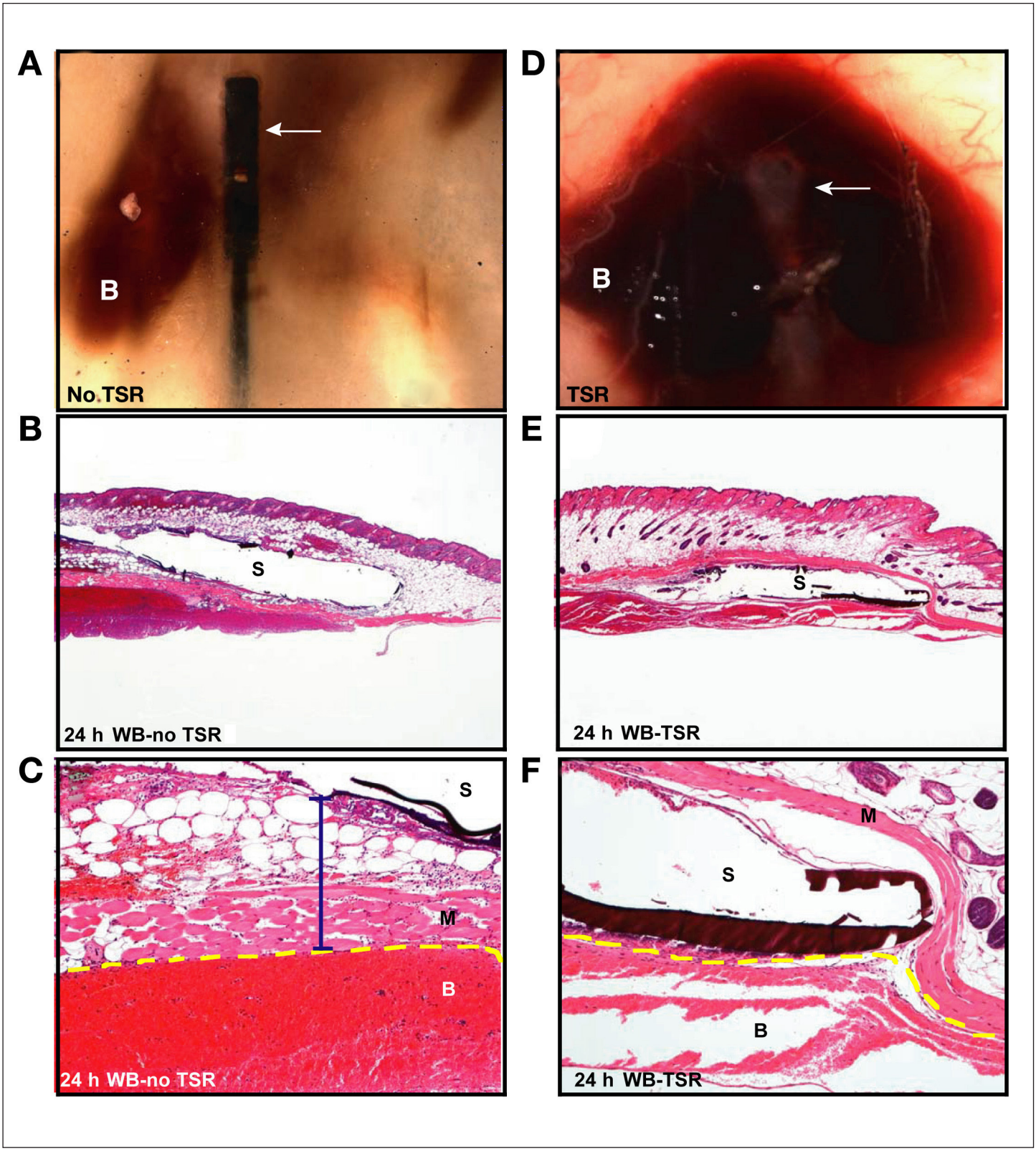

Figure 6. Morphologic and histopathologic evaluation of the tissue reactions induced in ICR mice by implanted Navigator sensors. Continuous glucose sensor was initiated upon Navigator sensor implantation, and WB was injected 1 DPI. Sensor performance was grouped into TSR and no TSR occurrence after the termination of this study at 2 DPI. The tissue adjacent to the sensors implanted for 2 DPI were obtained and evaluated **(A, D)** morphologic and **(B, C, E, F)** histopathologic. The original sensor location is designated by "S," and blood and muscle layers are designated as "B" or "M," respectively. The yellow dotted line in C and F indicate the injected-blood–tissue interface. The solid line demonstrates the distance between the blood–tissue interface and the sensor.

sensor output. However, this phenomenon can only be seen if the glucose level is raised high enough. In this particular study, we administered the mouse a glucose bolus injection twice. The second glucose injection was administered 20 min past the first (**Figure 7B**).

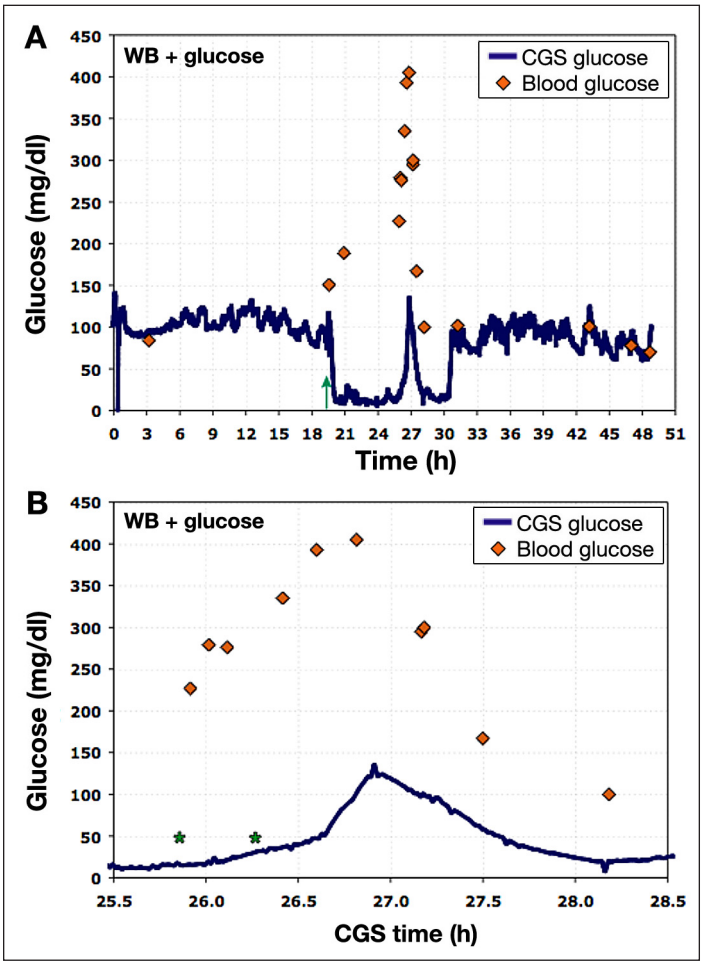

Figure 7. Impact of intraperitoneal injection of glucose sensor response during blood induced TSR in CD-1 mice. Continuous glucose sensor was initiated upon sensor implantation, and WB was injected at site of Navigator implantation 1 DPI. Intraperitoneal glucose bolus injection was administered approximately 5 h post-blood injection. **(B)** A magnified view of the data from A for the period 25.5–28.5 h after implantation. **(A)** The green error designates time of blood injection, and **(B)** green stars designate the time for glucose bolus injection. CGS, continuous glucose sensor.

Discussion

Significant effort and resources have been invested in developing subcutaneously implantable glucose sensors, and as a result, most commercial glucose sensors show good to excellent sensor performance for implantation periods of 3–7 days.[8–13] However, these commercial sensors still tend to be less accurate in the first 24 h post-implantation than in the subsequent days, and a contributing cause is periods of low sensor response, which are most common in the first 24 h post-implantation. The exact mechanism or substances responsible for these periods of reduced sensitivity are still unknown. It has been speculated that this initial delay in sensor functionality is the consequence of biofouling of sensors by tissue response to sensor implantation, possibly induced by influxing proteins and/or leukocytes,[14,15] but no definitive studies have confirmed or refuted this speculation. Currently, all methods of sensor implantation cause tissue trauma of varying types and degrees. Tissue hemorrhage and associated blood clot formation are commonly seen as a result of glucose sensor implantation and/or movement of sensors in the tissue. But virtually nothing is known about the impact of hemorrhage on sensor functionality *in vivo*.

In previous studies, we tested the hypothesis that blood and blood clots can directly interfere with GSF *in vitro*.[3] It was found that this interference is related to blood clot formation of WB, since neither HWB, serum, plasma-derived clots, nor total number of leukocytes interfered with GSF *in vitro* in the same way WB did. These *in vitro* studies supported the concept that the formation of hemorrhages at sites of glucose sensor implantation could have a major impact on GSF *in vivo*.

Based on these *in vitro* observations, we hypothesize that, *in vivo*, any local accumulation of RBCs, i.e., hemorrhages, at the sensing elements of implantable glucose sensors compromises the functionality of implanted sensors due to formation of a "metabolic sink," i.e., local cell metabolism of glucose within the microenvironment of the glucose-sensing element of the electrode. We further hypothesize that this *in vivo* RBC-based metabolic sink gives the appearance of loss of sensor functionality. To test this hypothesis, we first demonstrated that blood clots could induce loss of sensor function *in vitro* for two types of commercial glucose sensors (Abbott Diabetes Care Navigator and DexCom Seven). Next, we developed an *in vivo* mouse model that utilized direct blood injections at the tissue sites of senor implantation to mimic hemorrhages that can occur during sensor implantation and movement *in vivo*. Using the two types of sensors in the mouse model, we observed the same sharp decline in sensor response *in vivo* after direct blood injection at the site of sensor implantation. In general, these WB and HWB injection studies support the hypothesis that hemorrhaging (e.g., blood clots) at site of sensor tip can inhibit GSF *in vivo*. We further hypothesize that the reversal of the interference in GSF is the result of fibrinolysis, i.e., clot dissolution, that

liquefies the clot, ultimately returning sensor function *in vivo*.

Based on these findings, we believe a reasonable working model of the temporary reduced sensor response shortly after sensor implantation involves the following sequence of events:

Step 1. Sensor insertion, or movement once implanted, results in tissue damage.

Step 2. Tissue damage results in hemorrhage, releasing both RBCs and fibrinogen from the vasculature into the tissue space where the sensor is located, ultimately forming a blood clot around the sensor, including the glucose-sensing element, i.e., site of glucose oxidase location.

Step 3. The formation of the blood clots at the tip of the sensor (i.e., glucose-sensing region) results in fibrin-mediated "packing" of RBCs tightly to the sensor.

Step 4. The RBCs surrounding the microenvironment of the glucose-sensing element of the sensor begin metabolizing glucose, which results in a drop of interstitial glucose levels in this particular area relative to interstitial glucose levels distant from this site. Thus accumulation of RBCs near the sensor tip creates a metabolic sink, which consumes glucose, ultimately providing a glucose sensor reading quite different from the systemic blood glucose level.

Step 5. The RBC-induced loss of interstitial glucose levels at the sensing element of the sensor tip ultimately results in a drop of sensor output (i.e., nanoamperes). Depending on the location of the blood clot, size of the blood clot, and speed of clotting, there is variability in the appearance and duration of glucose metabolism and reduced signal phenomena.

Step 6. Liquefying of blood clots at sensor tip during the normal fibrinolytic process and drainage of the RBCs normally leads to an equalization of the local and systemic glucose concentrations, and a reliable sensor output.

Based on this model, any procedures that (1) prevent or minimize tissue trauma and hemorrhage, (2) protect the sensor tip from direct contact with the blood clots, and/or (3) speed the liquefaction of the blood clots will likely prevent or minimize the impact of blood clot formation and the subsequent temporary reduced sensor response seen in implantable glucose sensors as well as the type of implantable sensors.

As outlined in our studies, we also investigated the role of other cells and fluids in causing a TSR, including plasma (i.e., liquid, proteins, and platelets) injection. As described earlier, sensors exposed to plasma injection did not experience a TSR as compared with blood injection. We have concluded from these studies that intimate contact (short distances) between RBCs and sensors are needed in order to experience a TSR. Fibrin is also important since it maintains RBCs in close proximity with the sensing element. For the plasma study, we looked at the immediate acute tissue response to sensor implantation and the TSR in sensor output. We do not believe that recruited leukocytes are a factor in the *initial* TSR response since we did not see a significant accumulation of leukocytes within the short time frame (hours) post-blood injection. That said, it is quite possible that over longer timeframes, RBCs retained in the sensor implantation site could die and trigger inflammatory responses, which, in turn, could interfere with sensor function. Related to the direct impact of RBCs, fibrin, and RBCs + fibrin, we have previously conducted *in vitro* dye diffusion studies using a standard diffusion chamber and demonstrated that RBCs alone, fibrin alone, or the combination of RBCs + fibrin do not slow diffusion of small molecular weight dyes *in vitro*. Therefore, we believe that a mechanical blockade to glucose diffusion is unlikely *in vivo*, and as such, we do not think this to be the explanation to TSR. Rather, we believe that glucose metabolism of RBCs at the site of sensor-working electrode is the major cause of TSR.

Our current studies underscore the importance of (1) close contact of RBCs with the sensor at the implantation site and (2) the likely importance of fibrin clots in maintaining RBCs in close contact with the sensor. From clinical experience, we know that, despite bleeding occurring at the time of sensor insertion, a transient loss in sensor function is not always seen (e.g., TSR). Indeed, our animal experiment shows the same phenomenon that not every blood injection at the site of sensor location will lead to a temporary steady sensor signal loss over time. From our mice histological evaluations we concluded in order to experience a transient sensor signal loss upon blood injection, the blood clot needs to form in direct contact with the working electrode surface (e.g., location of enzyme-sensing layer). Occasionally, although it appears that the blood is injected precisely at the location of the working electrode (tip of the sensor), later histological evaluation determined that the blood clot formed at

a different location other than the sensing surface and, in some instances, only a few micrometers away from the working electrode. For example, in the case that blood clot formation occurs either on the shaft of the sensor or even at the working electrode but is separated from the working electrode by a layer of fat tissue, a temporary sensor signal reduction will not occur. Furthermore, in our studies, we showed only data for an injected volume of 50 µl; however, we also conducted experiments in which we injected less blood, and we were still able to demonstrate TSR. *Vice versa*, higher volume of injected blood (e.g., beyond 50 µl) or repeated blood injections do not necessarily lead to TSR. As such, we concluded from our histological evaluation that the amount of blood injected is not as critical as long as a blood clot forms in direct contact with the working electrode surface. In order to experience a TSR event, a cascade of events needs to happen, namely, bleeding at sensor site followed by immediate blood clot formation, and the blood clot needs to be in direct contact with the working electrode. As such, since the TSR event has to follow a precise cascade of events, bleeding alone at the site of sensor location does not necessarily have to lead to TSR.

Our data demonstrate significant instances (**Figures 3B**, **3E**, **3F**, **5D**, **5E**, and **5F**) where the injection of a small quantity of fluid (e.g., saline, plasma, WB, or HWB) near the sensor corresponds to an increased reference blood glucose level. We believe the systematic glucose increase is caused by the stress to the animal from the injection procedure, detailed as follows. Prior to injecting the fluid, the mouse is put under anesthesia and the mesh around the sensor is removed. Handling of the animal often causes stress-elevated blood glucose levels, which is intensified particularly for blood injections (e.g., HWB or WB) since the blood is usually obtained from the mouse, which will receive the fluid injection at the site of sensor implantation. Any blood drawing adds an additional stress factor to the animal because of the increased time component that the mouse is under isoflurane (anesthesia). Therefore, the measured blood glucose levels are often higher in mice undergoing any procedure than under regular housing condition due to the stress related to simply handling the animal, placing it under anesthesia, blood drawing, and injections of the various fluids.

Conclusion

Our present studies clearly demonstrate that the induction of hemorrhages at the site of glucose sensor implantation results in the accumulation or "pooling" of metabolically active RBCs to the sensing element of glucose sensors. These metabolically active RBCs that surround the sensors consume glucose diffusing to the sensor, which results in a "metabolic sink" that prevents diffusion of interstitial glucose from reaching the sensing element, thereby resulting in a TSR. This TSR appears as a drop of signal output. Therefore, the sensor is correctly reporting the glucose levels in the immediate vicinity of the sensing element, but not the interstitial glucose level outside the RBC metabolic barrier surrounding the sensing element of the implanted sensor. It should also be noted that damage to small vessels and, as such, the creation of tissue hemorrhage can occur not only during initial device implantation, but also as the result of mechanical movement of the sensor at any time after sensor insertion (i.e., post-insertion). Finally, these studies underscore the impact of accumulation of metabolically active cells (such as RBCs or inflammatory cells) at sites of sensor implantation, which can result in an unexplained or unexpected drop of sensor output, i.e., TSR. Further studies to better understand and prevent these TSRs should be undertaken in the future.

Funding:

Abbott Diabetes Care, Alameda, CA, provided funding for this research.

Acknowledgments:

We acknowledge Ms. Manjot Kaur and Dr. Yi Qiao for their technical assistance during these studies.

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