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Detecting Establishment of Shared Blood Supply in Parabiotic Mice by Caudal Vein Glucose Injection

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TITLE:

Detecting Establishment of Shared Blood Supply in Parabiotic Mice by Caudal Vein Glucose Injection

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KEYWORDS:

parabiosis, caudal vein injection, blood glucose, blood supply, donor mice, recipient mice

SUMMARY:

Here we describe a new method of detecting successful establishment of shared blood circulation of two parabionts through a caudal vein injection of glucose, which causes minimal damage and is not fatal to the parabionts.

ABSTRACT:

Parabiosis is an experimental method for surgically combining two parallel animals along the longitudinal axis of the body. We present a protocol for detecting the successful establishment of blood chimerism in parabionts by a caudal vein injection of glucose. Parabiotic mice were constructed. Glucose was injected into the donor mouse through the tail vein, and the fluctuation of blood glucose level was measured in both mice using a blood glucometer at different time points. Our results showed that after glucose injection, the blood glucose level in donor mice increased sharply after 1 min and decreased slowly thereafter. Meanwhile, the blood glucose level of the recipient mice peaked 15 min after injection. Similar results were obtained with Evans blue, used as a positive control for glucose. The synchronous fluctuation of blood glucose levels indicates

that blood flow between the two mice was established successfully.

INTRODUCTION:

Parabiosis is a modeling method in which two living organisms are joined together surgically and develop as a single physiological system with a shared circulatory system[1]. Such models have been widely used for studying physiology owing to the advantage that the substances produced by a single individual can act on both animals at the same time via the shared circulatory system. Since the mid-1800s when parabiotic experiments were pioneered by Paul Bert[2], the methods for constructing parabiotic models have become standardized. However, a straightforward and convenient method for verifying the successful establishment of blood chimerism has been missing. It has been reported that cross-circulation can be successfully assessed by intraperitoneally injecting 0.5% Evans blue dye in one of the parabionts followed by measurement of the absorbance of Evans blue in the blood of both parabionts with a microplate reader[3]. Another method requires a specific mouse breed that contains $CD45.1^+$- and $CD45.2^+$-labeled monocytes in each parabiont. Cell cytometry is then used to determine blood chimerism by measuring the frequency of the two markers in monocytes from spleen or blood[4]. However, these methods are often lethal or cumbersome to the animals, and a safe and simple method for quick and reliable verification of parabiotic models is highly desirable. In this study, we established a new method for this purpose, which was validated in a mouse model of parabiosis. Glucose concentration in blood samples drawn from a tail vein is measured using a glucometer, and the pattern of changes of glucose level in donor and recipient mice is considered an indication of circulation chimerism. We named this method the "glucose fluctuation method". The application of this validation method is not limited to mice but could be extended to diverse pathological models except for those with serious dysregulation of glucose metabolism. The procedure is simple, timesaving, and safe.

PROTOCOL:

All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee of Harbin Medical University.

NOTE: The tools and equipment required for the method are listed in the **Table of Materials**.

1. Preparation of materials and animals

1.1. Order C57BL/6 male mice with weight between 20 g–25 g from a standard laboratory animal supplier.

1.2. House the mice in a cycle of 12 h light: 12 h darkness at 24−26 °C with ad libitum access to water and food.

2. Parabiosis

2.1. Anesthetize the mice by intraperitoneal injection of 20 g/L 2,2,2-tribromoethanol at a concentration of 0.1 mL/10 g. Confirm proper anesthetization as indicated by muscle relaxation, slow and steady breathing, loss of skin stimulation reflex, and disappearance of corneal reflex.

2.2. Perform the parabiosis procedure as previously described[5].

2.2.1. Place the mice in the supine position. Thoroughly shave the left side of one mouse and the right side of the other mouse starting at approximately 1 cm above the elbow to 1 cm below the knee with an electric shaver.

2.2.2. Create longitudinal skin incisions starting from 0.5 cm above the elbow to 0.5 cm below the knee joint using a pair of sharp scissors on each animal's shaved side.

2.2.3. Detach the skin from the subcutaneous fascia gently following the incision.

2.2.4. Connect the olecranon and knee of the parabiont with a 3–0 suture.

2.2.5. Suture the shaved skin with a continuous 5–0 suture.

2.3. Inject 0.5 mL of 0.9% NaCl subcutaneously to each mouse to prevent dehydration.

2.4. Inject penicillin (50,000 IU/day) subcutaneously to each mouse to prevent inflammation for 3 days.

2.5. Inject tramadol (10 mg/25 g/day) intramuscularly to each mouse to relieve pain for 3 days.

3. Validation of circulation chimerism

3.1. Glucose fluctuation method

NOTE: Verify the successful construction of circulation chimerism between parabionts using the glucose fluctuation method (no fasting) on the 10th day after parabiosis surgery.

3.1.1. Anesthetize the parabionts by intraperitoneal injection of 20 g/L 2,2,2-tribromoethanol to each mouse at a concentration of 0.1 mL/10 g.

3.1.2. Rub the caudal veins in the flank of one of the parabiont's tail with a cotton ball soaked in 70−75% alcohol to clean the tail and dilate the blood vessels.

3.1.3. Hold a 2.5 mL syringe (No. 5 needle) containing glucose in the right hand and keep the needle parallel to the vein (less than 15°).

3.1.4. Insert the needle at a position approximately 2−4 cm from the tail tip.

3.1.5. Inject 100 µL of glucose (1.2 g/kg) into the donor within 10 s.

NOTE: The term donor refers to the parabiont that receives the glucose injection through the tail vein. The recipient is the other parabiont, which does not receive glucose directly.

3.1.6. Cut off the tail tip of the donor and recipient mice with a scissor and collect a drop of blood at different time points after the injection of glucose (1 min, 5 min, 10 min, 15 min, 20 min, 30 min, 40 min, 50 min, and 60 min).

3.1.7. Drip the blood into the center of glucometer test strips for glucose level detection.

3.2. Use Evans blue dye as a positive control[3].

3.2.1. Inject 200 µL of 0.5% Evans blue dye intraperitoneally into the donor mouse.

3.2.2. Anesthetize the parabionts by intraperitoneal injection of 20 g/L 2,2,2-tribromoethanol to each mouse at a concentration of 0.1 mL/10 g.

3.2.3. Collect blood from both parabionts by cardiac puncture 2 h later.

3.2.4. Centrifuge the blood samples at 916 x *g* for 15 min.

3.2.5. Collect serum from the supernatant.

3.2.6. Dilute the serum with 0.9% NaCl at 1:50.

3.2.7. Measure the absorbance of the diluted serum samples at 620 nm with a spectrophotometer.

REPRESENTATIVE RESULTS:

In six donor mice, blood glucose levels sharply increased to 26.5 µmol/L (173% increase) at an average of 1 min after the injection of 100 µL of glucose (1.2 g/kg) through the tail vein and then gradually decreased to 13.3 µmol/L at 60 min. In recipient mice, blood glucose slowly increased after injection and reached the first peak level at 15 min (47% increase, 12.2 µmol/L). Based on the above results, the standard for circulation chimerism was set as follows: 1) a sharp increase in the blood glucose level (a minimum 100% increase or >20 µmol/L) in donor mice within 1 min after glucose injection, and 2) a significant increase in the blood glucose level in recipient mice 15 min after injection (a minimum 37% increase) (**Figure 1**).

The concentration of Evans blue dye in the serum of parabionts also indicated the

successful construction of circulation chimerism (**Figure 2**). We euthanized the parabionts after blood glucose measurement using 5 mL of 2,2,2-tribromoethanol (20 g/L). The subcutaneous vascular junctions between the parabionts were clearly observed (**Figure 3**).

Supplementary Figure 1 shows that the donor mice had a significant blood glucose level increase 1 min after glucose injection, while the blood glucose level of the recipient mice was not elevated, which demonstrated that the circulation chimerism in parabionts was not successfully established 1 day after parabiosis surgery. Similarly, the blood OD level in recipient mice was not as elevated as that of donor mice (**Supplementary Figure 2)**.

Based on the results from the glucose fluctuation method, we found that two pairs of parabionts did not establish blood chimerism 15 days after parabiosis surgery. As shown in **Supplementary Table 1**, the two recipient mice did not have an increased blood glucose level within 60 min after glucose injection into the donor mice. The blood concentration of Evans blue in the two recipients was also not elevated (**Supplementary Table 2**), which demonstrated that the glucose fluctuation method was as sensitive as the Evans blue method.

Moreover, to evaluate the influence of injected glucose on insulin metabolism, we detected the blood insulin level 1 h and 3 h after injection of 100 μL of glucose (1.2 g/kg) in mice (**Supplementary Figure 3**). The blood insulin level was remarkably decreased 1 h after glucose injection because of quickly increased glucose and recovered to normal levels at 3 h. These results demonstrated that the effects of glucose we injected on insulin metabolism were restorable.

FIGURE AND TABLE LEGENDS:

Figure 1: Changes of blood glucose level in parabionts after injection of glucose through the caudal vein. (**A**) Blood glucose level of donor mice. (**B**) Blood glucose level of recipient mice (n = 6). The data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs. 0 min.

Figure 2: The concentration of Evans blue in serum samples of parabionts measured by a microplate reader. The data are presented as the mean ± SEM. ***p < 0.001 (n = 6).

Figure 3: Generation of subcutaneous vasoganglions in connected skin between the parabionts. Left: Representative image of the parabiosis mice. **Right:** A representative image of the subcutaneous vasoganglion between the parabionts.

Supplementary Figure 1: The blood glucose level of parabionts was tested 1 day after parabiosis surgery.

Supplementary Figure 2: The concentration of Evans blue measured by microplate

reader in the serum of the parabionts 1 day after parabiosis surgery.

Supplementary Figure 3: The concentration of insulin in the serum of the mice after glucose injection.

Supplementary Table 1: Blood glucose level (μmol/L) in parabionts 15 days after parabiosis surgery.

Supplementary Table 2: Blood concentration of Evans blue (OD value) in parabionts 15 days after parabiosis surgery.

DISCUSSION:

Parabiosis refers to the surgical technique of connecting two living animals to establish a common vascular system by experimental means[6–8]. The advantage of this model is that the substances produced by a single individual can act on both animals at the same time. Thus, the parabiosis model can be used to explore the role of a substance or factor in a related disease, producing many meaningful and innovative conclusions. In view of its great application value, the model has brought about a better understanding of cardiovascular system diseases[8–13], nervous system disorders[14,15], organ transplantation[16], and diabetes[17,18].

However, verification of the successful establishment of circulation chimerism is the first and determining step for studies with parabiosis. Current studies have reported some methods for detecting circulation chimerism. Loffredo et al.[4] reported that blood chimerism was confirmed in parabiotic pairs by measuring the mixed frequency of monocytes in the spleen with different labeled markers in the donor ($CD45.1^+$) and recipient ($CD45.2^+$) mice. In this method, specific mice with $CD45.1^+$- or $CD45.2^+$-labeled monocytes for each parabiont were used for parabiosis. Cell cytometry was needed to determine blood chimerism by measuring the integration of the marked blood cells. In addition, Marta et al.[3] assessed cross-circulation by intraperitoneally injecting 200 μL of 0.5% Evans blue dye in one of the parabionts. Blood from both parabionts was collected 2 h later by cardiac puncture. Blood chimerism was determined by an increased Evans blue concentration in the recipient mice, which was tested by a microplate reader. Although these strategies allow us to determine blood chimerism, there are still many limitations that cannot be ignored. First, for lethal methods, the blood chimerism can only be confirmed at execution. However, in our method, the establishment of blood chimerism can be tested any time after parabiosis surgery. Parabionts with unsuccessful established circulation chimerism can be excluded for further study in advance to reduce unnecessary workload. Indeed, using our glucose fluctuation method we were able to pick out the mice with unsuccessful construction of blood chimerism in certain parabionts, which might be due to insufficient time of parabiosis, unstable surgery manipulation, delayed wound healing, or disconnected body tissue caused by animal struggling. In such circumstances, the failure of blood chimerism was also confirmed by using the Evans blue method. These results indicate that the glucose fluctuation method was as effective as

the Evans blue method (**Supplementary Figure 1** and **Supplementary Figure 2, Supplementary Table 1** and **Supplementary Table 2**). Second, the currently used methods of validation are excessively time-consuming and difficult, as opposed to this new method, which is simple and effective.

In the present study, we successfully tested for circulation chimerism in parabiotic mice by the glucose fluctuation method. The mice were anesthetized for the glucose injection and blood glucose level measurements, which made their manipulation easier. We injected 100 μL of glucose (1.2 g/kg) through the tail vein of the mice within 10 s. Under these conditions, a regular fluctuation of blood glucose levels in donor and recipient mice was observed. Importantly, the amount of glucose we used tended to stably elevate the blood glucose level within a certain range. Moreover, the results showed that the blood insulin level recovered to the normal range 3 h after glucose injection (**Supplementary Figure 3**), suggesting that the amount of glucose injected into the mice had minimal effects on insulin metabolism. In addition, the dosage of glucose we gave to the donor was lower than that used for the glucose tolerance test of mice (for the GTT, 2 g/kg glucose is intraperitoneally injected to the mice, equaling approximately 1.6 g/kg through caudal vein injection[19–21]), which means that the dosage of glucose for the glucose fluctuation method would not cause hyperglycemia and other damaging alterations. In conclusion, the method we used is effective, harmless, and timesaving. However, it might be limited to diabetic mice in parabiosis, which still need further experiments for confirmation.

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DISCLOSURES:

The authors have nothing to disclose.

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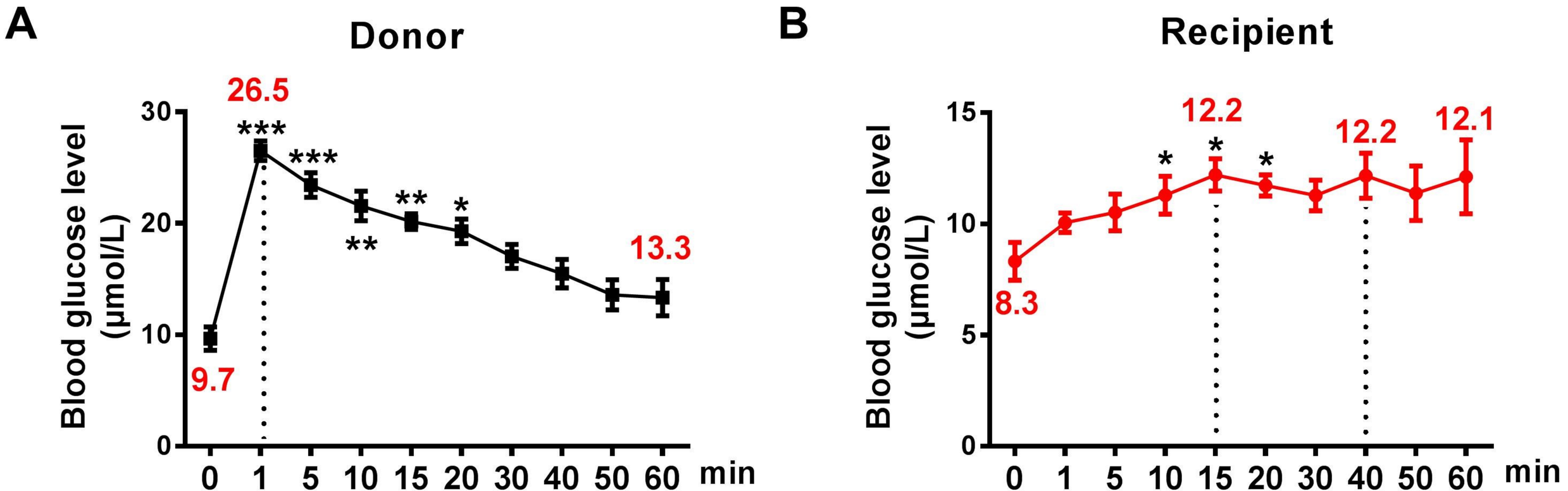

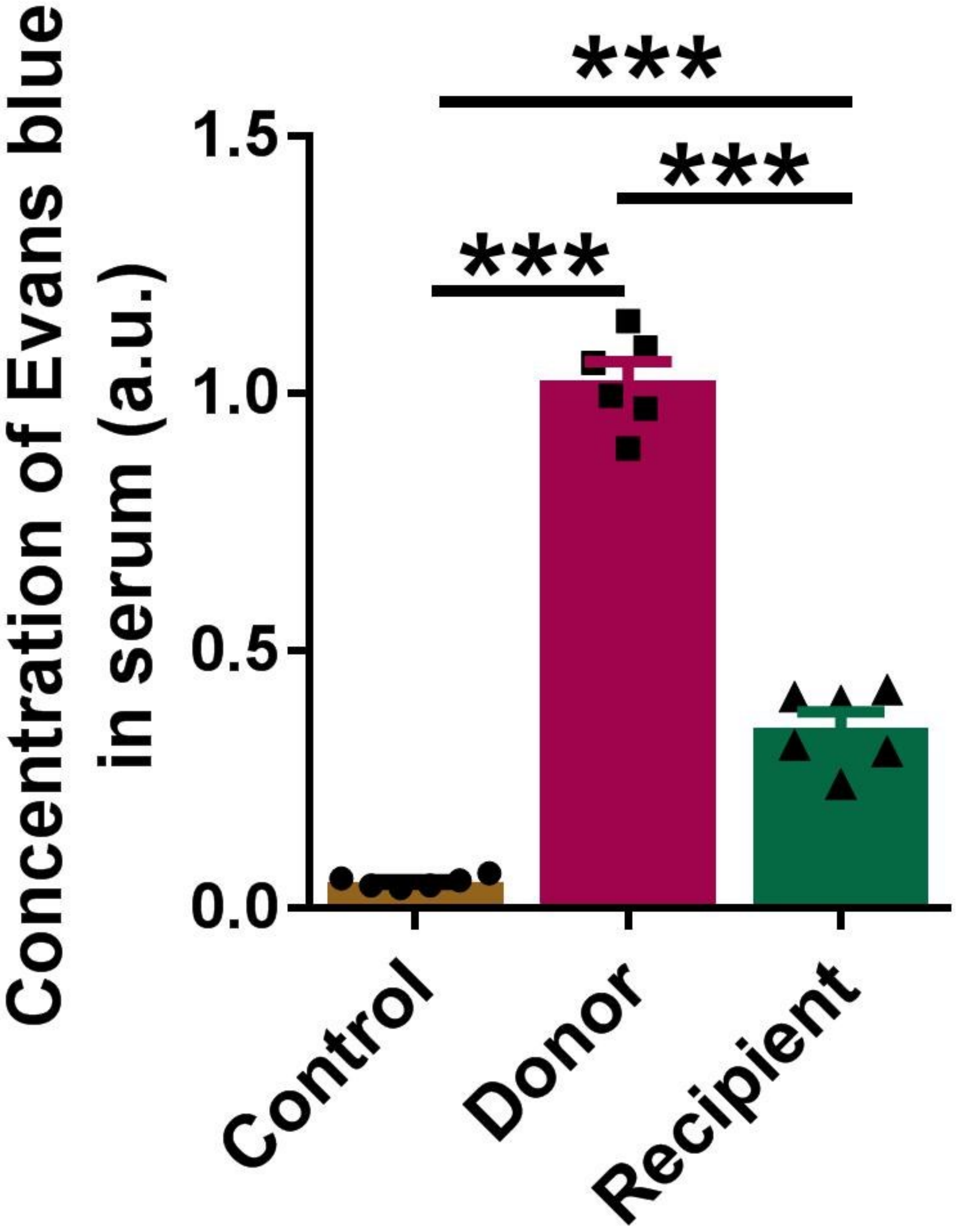

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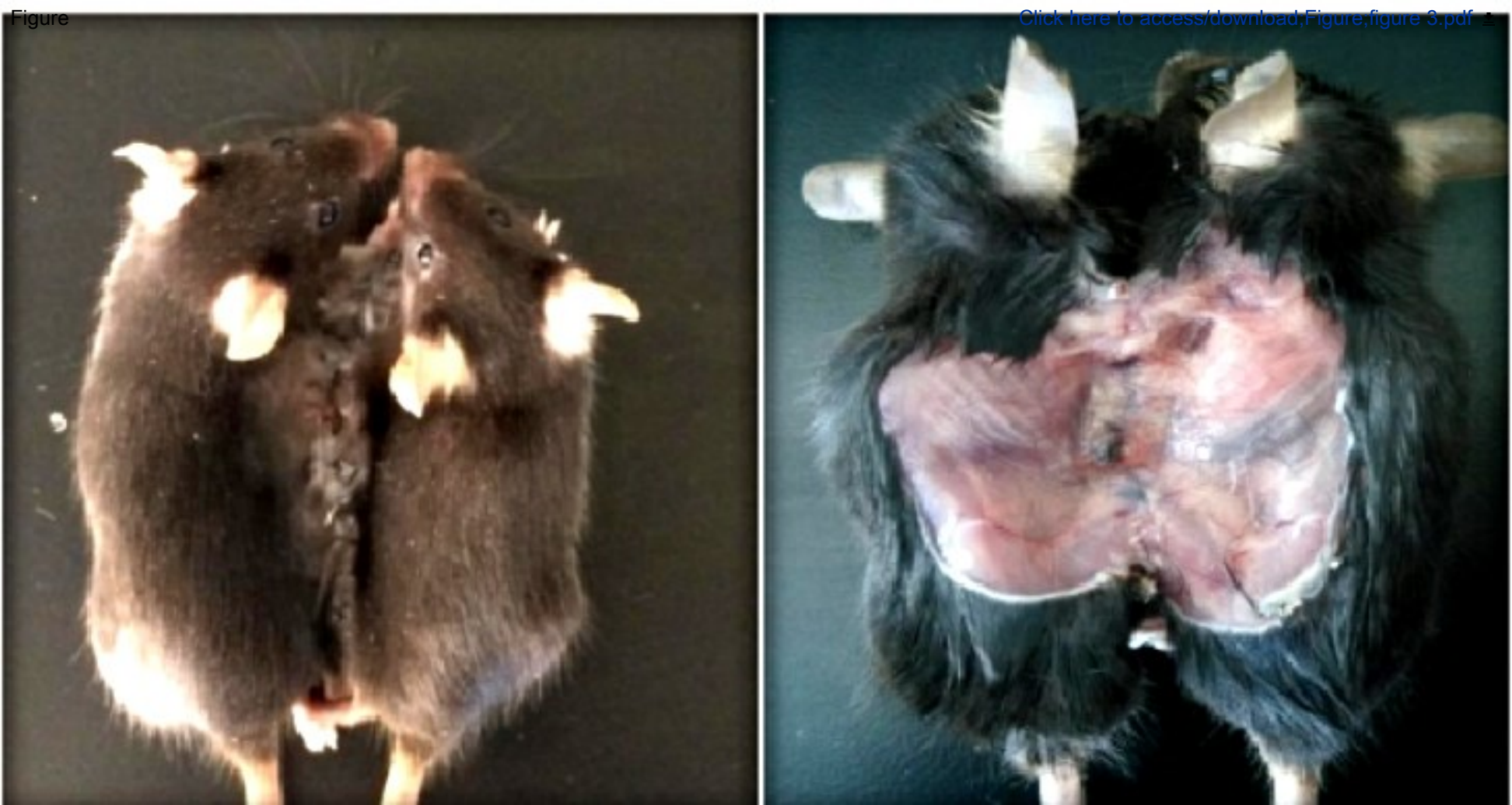

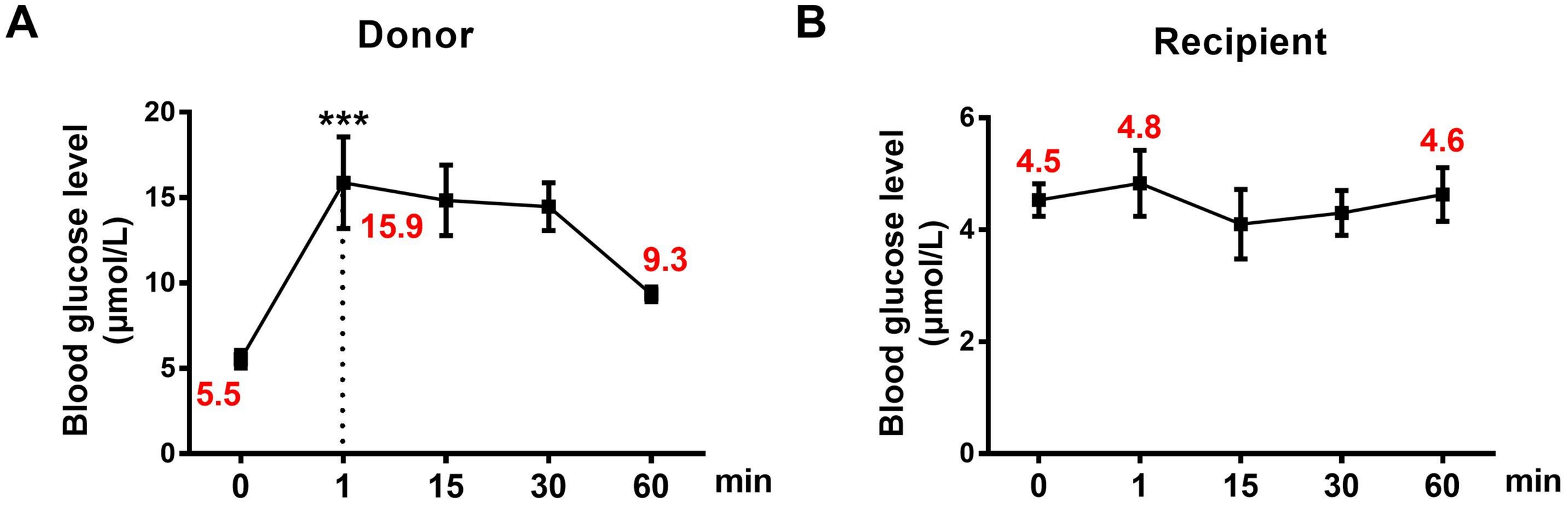

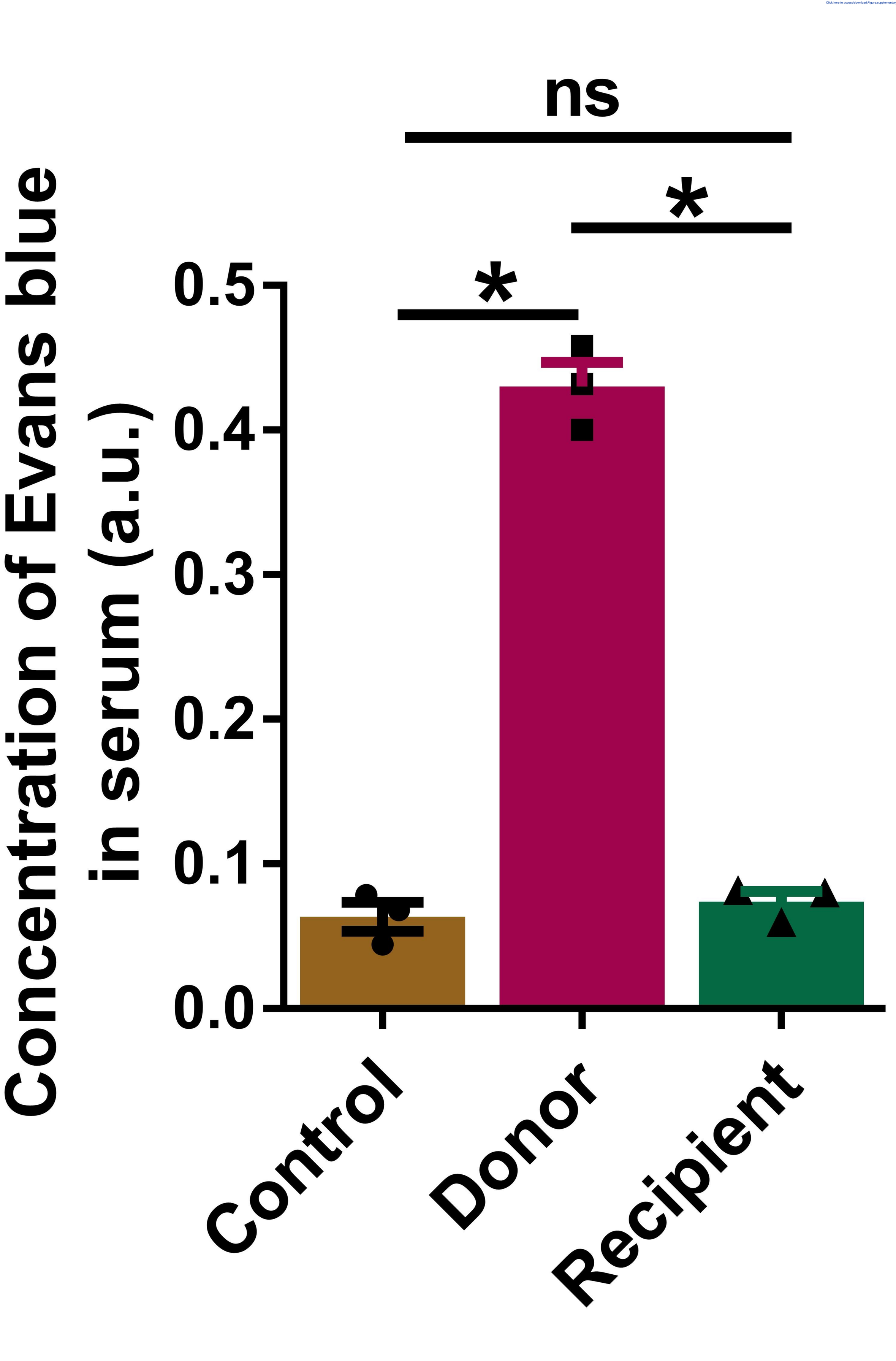

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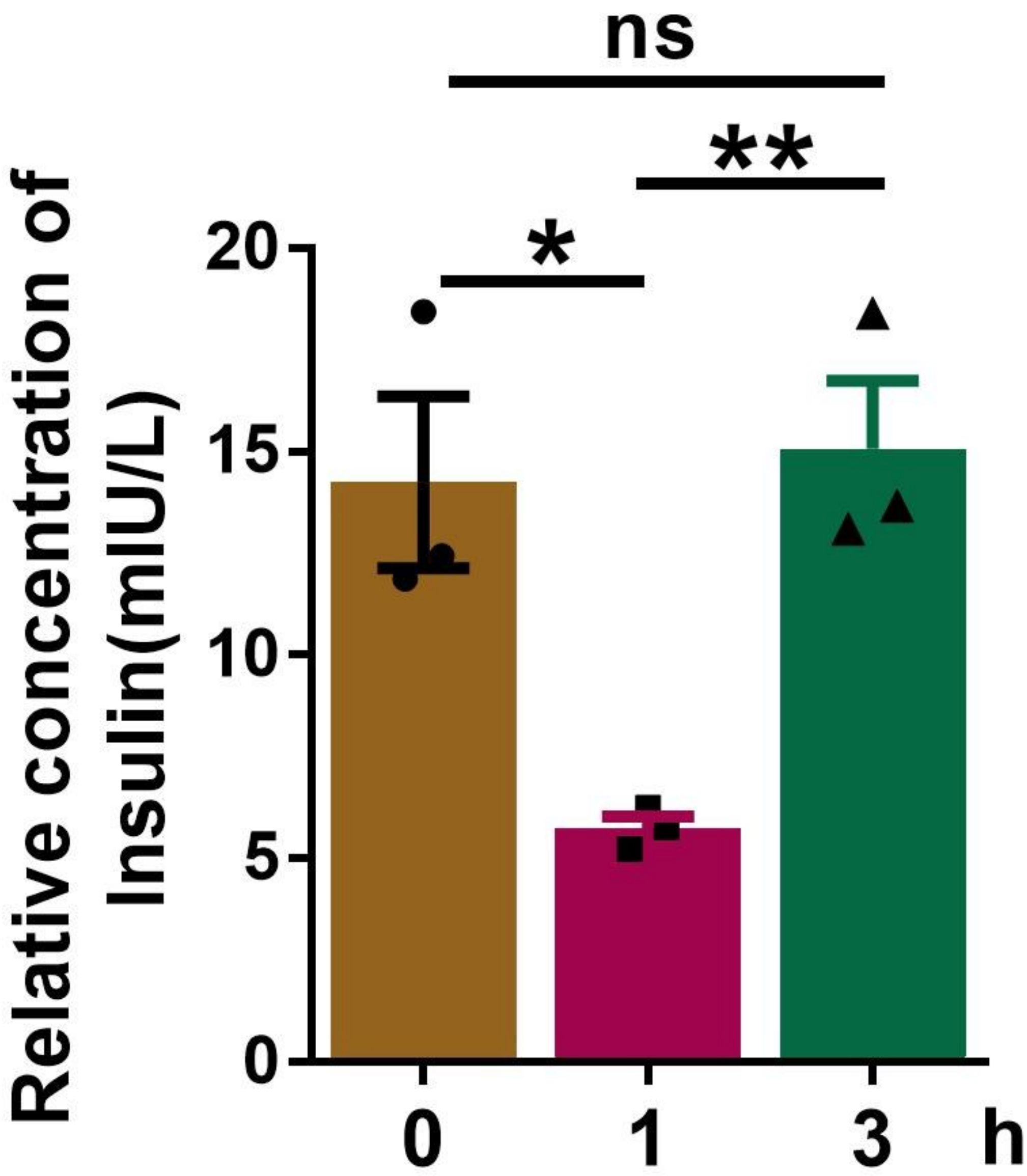

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parabiosis surgery

blood glucose concentration detection

blood insulin concentration detection

parabiosis surgery

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Changes to be made by the author(s):

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

We very much appreciate for sending us the editorial reports and giving us the opportunity to revise and improve our manuscript before making an editorial decision.

Our revised manuscript has been resubmitted for your further consideration for publication in *JOVE*. The editorial comments have been addressed as detailed in a point-by-point manner below. The modifications are indicated with red font in the revised manuscript.

2. Please revise lines 48-49 and 51-53 to avoid previously published text.

We have revised the text to make it different from what has been published.

3. Please address specific comments marked in the attached manuscript.

We have made corresponding revisions according to each specific comments marked in the manuscript.

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7. Please include a short title and a short description of each supplemental figure/table

at the end of the Representative Results in the manuscript text.

As your suggestion, we included a short title and a short description of each supplemental figure/table at the end of the Representative Results in the revised manuscript text.

Supplementary figure 1

The blood glucose level of parabionts 1 day after parabiosis surgery was tested (Supplementary figure 1). The results showed that the donor mice had a significant increase of blood glucose level 1 min after glucose injection, while the blood glucose level of recipient mice was not elevated. These results demonstrated that the circulation chimerism in parabionts was not successfully established 1 day after parabiosis surgery.

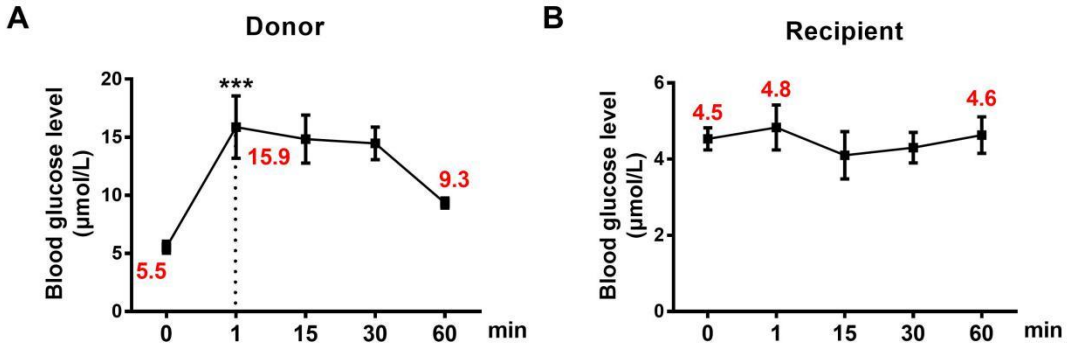

Figure 1. Blood glucose level of donor and recipient mice 1 day after parabiosis surgery. n=3. The data are presented as the mean±S.E.M. ***p<0.001 vs. 0 min.

Supplementary figure 2

Evans blue method was also used (Supplementary figure 2). Similarity, the blood OD level in recipient mice was not elevated as donor mice did. These results demonstrated that the glucose fluctuation method was as effective as Evans blue method.

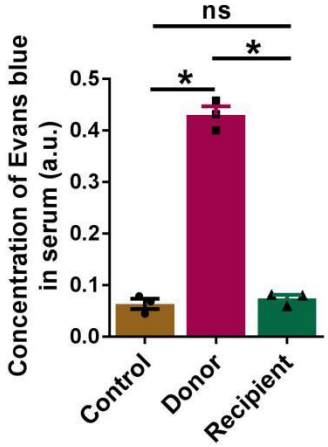

Figure 2. The concentration of Evans blue in the serum of the parabionts 1 day after parabiosis surgery was measured by microplate reader. n=3. The data are presented as the mean±S.E.M. *p<0.05, ns presents no significance between the two groups.

Supplementary Table 1 & 2

We found two pairs of parabionts didn't establish blood chimerism 15 days after parabiosis surgery, which was tested by glucose fluctuation method and Evans blue

method. As shown by the following results, the two recipient mice didn't have an increased blood glucose level within 60 min after glucose injection into donor mice (Supplementary Table 1). Similarity, the blood concentration of Evans blue in recipients was also not elevated as shown in donor mice (Supplementary Table 2). These results demonstrated that glucose fluctuation method was as effective as Evans blue method.

Glucose fluctuation method:

Table 1. Blood glucose level (μmol/L) in parabionts 15 days after parabiosis surgery

	0 min	5 min	15 min	20 min	40 min	60 min
Donor-1	5.7	26.2	21.2	17.6	16.9	15.4
Recipient-1	6.7	5.8	5.9	6.2	5.2	5.4
Donor-2	8.4	25.5	21.1	20.5	17.4	13.8
Recipient-2	6.7	5.8	5.9	6.2	5.2	5.4

Evans blue method:

Table 2. Blood concentration of Evans blue (OD value) in parabionts 15 days after parabiosis surgery

	Control-1	Donor-1	Recipient-1	Control-2	Donor-2	Recipient-2
OD value	0.059	0.935	0.062	0.068	0.862	0.073

Supplementary figure 3

We tested the blood insulin level in mice 1h and 3h after injection of 100 μL of glucose (1.2 g/kg) into tail vein (Supplementary figure 3). We found the blood insulin level was remarkably decreased 1 h after glucose injection because of quickly increased glucose, while recovered to normal level at 3 h. These results demonstrated that the effects of glucose we injected on insulin metabolism was restorable.

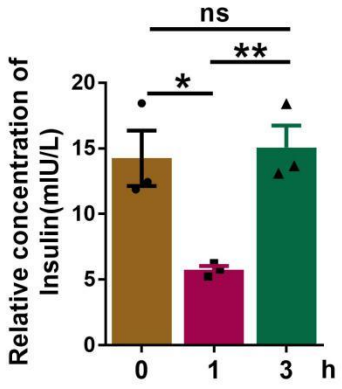

Figure 3. The concentration of insulin in serum of mice after glucose injection. n=3. The data are presented as the mean±S.E.M. *p<0.05, **p<0.01. ns presents no significance between the two groups.

Supplemental Table 1

	0 min	5 min	15 min	20 min	40 min	60 min
Donor-1	5.7	26.2	21.2	17.6	16.9	15.4
Recipient-1	6.7	5.8	5.9	6.2	5.2	5.4
Donor-2	8.4	25.5	21.1	20.5	17.4	13.8
Recipient-2	6.7	5.8	5.9	6.2	5.2	5.4

Supplemental Table 2

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	Control-1	Donor-1	Recipient-1	Control-2	Donor-2	Recipient-2
OD value	0.059	0.935	0.062	0.068	0.862	0.073