Western Blot to Evaluate Infection Level Incurred by ECMO (Pilot Study)

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Abstract

ECMO inevitably increases the risk of infection over time; therefore, lowering infections acquired during ECMO should be further investigated and developed accordingly (Bizzarro, 2011). The degree of dysfunction by ECMO is evaluated using several methods, namely: ultrastructural changes in gut epithelium by TEM, plasma lipopolysaccharide (LPS) levels of bacterial products after receiving ECMO, etc. In particular, bacterial lipoteichoic acid (LTA) antigens in plasma can be measured over time by Western blot laboratory technique in association with the loss of gut barrier function below. The purpose of this pilot study is to evaluate how newly-developed ECMO reduces infection levels by controlling and stabilizing the overall hemodynamic status, based on the outcome of a Western blot.

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Protocol

(A) Cell Culture (SubCulture + Cell Counting)

Step 1.

Media: RPMI1640 to provide nutrition required for cell growth and to neutralize the trypsin
- Warm the media at 37 for 30 min;
- Prepare the trypsin (w/ EDTA) and the PBS;

- Suck away the media from the flask and then wash the cells (HT-29) with PBS (T- 25 = 5mL, T-75=10mL);
- Treat the cells with 1.0 mL of trypsin (w/ EDTA) and incubate the flask at 37 for 5 min in order to separate the cells from the flask floor;

- Prepare the materials (6 dishes, one 1.5mL tube for cell counting, 1 flask for cell subculture);
- After the incubation, add 4.5mL media to the previous flask;
- Take 5mL of the cells in the cube into the 15mL tube for centrifugation for 3 min at 1000RPM;

- After centrifugation, remove the media (in case of cell counting, re-suspend the cells with 1mL of media, take 50uL and then proceed with cell counting);
- Afterwards, add 2mL of media, do pipetting up and down;
- For culturing, place 6.0*105 cells/disk on each dish and a new flask for subculture. Then, add media up to 5mL in total to each dish and T-25 (when using T-75 flask, add media up to 10mL in total).

(Although it is the standard method to count the cells in a flask per a protocol using lattice, it is also somewhat acceptable to get ball-park number for cell numbers under microscope. Although this procedure provides only a rough estimation of the total number of cells, it may not affect the results of Western blotting much, since, at the Quantification step, we can calibrate and even out the protein amount of each flask or dish.)

(B) Cell Stimulation and Protein Extraction

Step 2.

- Treat the HT-29 cells with 6μM of TNF-α or LPS (50ng/mL) (in each dish for the purpose of cell stimulation and incubate for 0h, 1/6h, 1/2h, 1h, and 2h each, accordingly);
- Prepare ice in a styrofoam box;

- Discard media and wash cells with 5 mL of PBS;
- Harvest cells (in 1.5mL of PBS) and centrifugate at 30005000 RPM for 10 min at 4°C ;
- Remove PBS and add 5080μL of lysis buffer based upon the amount of centrifuged cells;
- Maintain constant agitation (or vortexing of the tube every 5min) for 30min at 4°C or at ice box;
- Centrifugate at 13200 RPM for 15 min at 4°C;;
- Transfer supernatant of each tube into its corresponding new 1.5mL tube.

(C) Western Blotting

Step 3.

Similar to Southern blotting, Western blotting employs gel electrophoresis to separate materials, perform a membrane transfer procedure, and then detects to locate the specific biologic product in need. However, Western blotting detects protein instead of DNA segment and uses antibody instead of complementary DNA segment probe; it also and employs acrylamide gel instead of agar gel.

(C-1) Making a Running Gel (10% Running Gel)

Step 4.

- Prepare the electrophoresis kit and fill the chamber with DW for detecting any possible fluid leakage from the kit;

- Prepare a 50mL tube for running gel and a 15mL tube for stacking gel;
- Make mixture according to the instruction (both running gel and stacking gel), by adding reagents for an acrylamide gel polymerization reaction in the order specified by the manufacturer-specific guidelines;

(a) 10% Running Gel Solution (20mL)	(b) 5% Running Gel Solution (6mL)
7.9mL DDH2O 6.7mL 30% Acrylamide Mix 5mL 1.5M Tris Ph8.8 0.2mL 10% SDS	4.1mL DDH2O 1mL 30% Acrylamide Mix 0.75mL 1.0M Tris Ph6.8 0.06mL 10% SDS
(Add the followings immediately before use) 0.2mL 10% APS 0.008mL TEMED	(Add the followings immediately before use) 0.06mL 10% APS 0.006mL TEMED

- Prepare the 10% Running Gel Solution per the aforementioned protocol. (Note that APS & TEMED must be added immediately before adding the solution to the cassette.)

- Place dry short plate in the front of spacer plate in the casting frame with the arrows on spacer plate facing up.

- Place casting frame in the casting stand.
- Now, sequentially add APS and TEMED. After stirring the solution, carefully fill the chamber (or cassette) with a proper amount of running gel mixture;
- Add 250uL of isopropanol to the top of the running gel to even out the surface of running gel and leave it to harden.

(C-2) Making a Stacking Gel

Step 5.

- Wash isopropanol on top of running gel of the kit;
- Check whether the running gel has been polymerized. If so, remove the isopropanol layer on top of the running gel.
- Dry out the chamber and add sequentially APS and TEMED to the gel mixtures;
- After gently stirring the solution, carefully fill the stacking gel on top of the hardened running gel and install a comb between the two glass plates (The comb should be carefully and obliquely slided down into the running gel between the two plates in order to prevent formation of a bubble in-between the comb and gel by inserting the comb too quickly into the gel.)
- After the gel gets hardened, very carefully remove the comb.
- Place the sample in wells and proceed to electrophoresis.

(C-3) Sample Protein Quantification

Step 6.

- Mix and vortex 530uL Bradford (5X) solution and 530*4 uL of DW;
- Load 200uL of the mixture, (0, 1, 2, 3, 4, 5)uL of BSA (1mg/mL), and 1uL of samples in quantification kit; pipette up and down to mix the protein with Brandford mix; then, remove bubbles with a needle;
- Perform the Quantification accordingly.
:: On the 96 Well-Plate, load 200uL of the mixture each, and then add the following accordingly:
0uL BSA + 1uL BSA + 2uL BSA
3uL BSA + 4uL BSA + 5uL BSA
1uL sample I + 1uL sample II + 1uL sample III
1uL sample IV + 1uL sample V

(C-4) Sample Preparation

Step 7.

- Add 5x sample buffer, lysis buffer, and the calculated amount of sample into 600uL tubes, according to the quantification calculation;

- Place tubes in the boiled water for 10 min;
- Cool the tubes in the ice.

(C-5) Gel Electrophoresis

Step 8.

- make 1X EP buffer (100mL 5X EP buffer + 400mL DW water)

- Wash the glass with DW, carefully remove the combs, and remove the bubbles with DW;
- Assemble electrophoresis kits and pour the 1X EP buffer between the plates;
- Pour 1X EP buffer into the kit (check whether 1X EP buffer leaks out into the tank); - Load samples and run electrophoresis;
- Make 1X transfer buffer (100mL 10X transfer buffer + 200mL EtOH + 700mL DW) immediately and store at -20°C, while running electrophoresis;
- When electrophoresis is almost finished, activate PVDF membrane by immersing it in MeOH for 1 minute.

(C-6) Transfer

Step 9.

- Prepare transfer kits, tray, and 1X transfer buffer;

- Place the following materials in the order of bottom to top (Make sure that the concaved surface of the PVDF membrane faces the gel.):
Plate – Sponge – Filter paper – Gel – Membrane – Filter Paper – Sponge – Plate
- Mount the plates into the tank kit and fill the tank with transfer buffer;
- Run the kit at 100V for 8090 min;
- After transfer, cut the membrane into appropriate sizes.

(C-7) ImmunoBlot

Step 10.

- Add Ponceau solution (5uL of Ponceau + 6mL of DW) in order to check the bands with bare eyes;

- Wash Ponceau solution with DW and TBST (Tris Buffered Saline with Tween) several times;

- Block the membrane with 5% of skim milk for 1hr;
- Wash the membrane with 10mL of TBST for 5 minutes, 3 times in total;
- Add 1.5uL first antibody diluted in 6mL of 5% BSA (1:4000) + incubate for 2hr;
- Rinse 3 times with 10mL of TBST + incubate 5 min each time;
- Add 1uL of second antibody diluted in 6mL of 5% BSA (1:6000) + incubate for 1hr; - Rinse 3 times with 10mL of TBST + incubate 5 min each time.

(C-8) Development

Step 11.

- Prepare the developing Casette and ECL kit;

- Place the membrane of the Casettte; then, rinse the membrane with DW and TBST;
- Cautiously remove the wash buffer around the membrane with a paper towel; then, treat the membrane with ECL solution (750mL of white bottle + 750mL of brown bottle of West-Q chemiluminescent Substrate Kit);
- Proceed developing.