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Renova Life's Bovine OPU-IVF Protocol

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SAR IVM

Expiration Date: If continuously stored at 4°C, two weeks after production date
If frozen upon arrival, 2-3 months at -20°C.
Renova Life, Inc. (RLI) recommends that SAR-IVM maturation be stored at -20°C upon arrival, this makes FSH/LH function as effectively as freshly prepared lots.

1. At the time of arrival from Renova, pipet 1 mL SAR-IVM into 1.8 mL cryovials and overlay with 100 µL of oil into 1ml vials and place immediately into -20°C freezer.
2. Remove one vial SAR IVM from freezer and place into 5% CO_2 incubator at 39 °C with the lid loose for equilibration overnight. One vial SAR IVM is for maturation of up to 25 or more oocytes per cow.
3. In the morning of OPU, remove vial(s) from incubator, screw cap on tight before removing from incubator and parafilm around cap, place immediately into 39 °C warmed transport incubator.
4. Maintain vials at 39 °C at all times.

Addendum:

*If oocytes will be aspirated and transported in under 2 h, oocytes can be stored in 1 mL vials of OWP (oocyte washing prior to IVM) media and held at 39 °C in transport incubator. At the lab each cow's oocytes should be washed through one 50 µL drop of SAR-IVM before placing into SAR-IVM drop for maturation. For example: 1 cow = 1 (50 µL) drop for washing + 1 (50 µL) drop for maturation culture.

*If oocytes will be aspirated and transported in less than 6 h, oocytes can be transferred into SAR IVM pre-equilibrated media dishes (25 oocytes per 50uL drop). Dishes need to be pre-equilibrated for 2-3 hours at 5% CO_2, 39 °C.

* SAR IVM vials can be used within 48 h of CO_2 equilibration. If this is the case, vials should be equilibrated overnight in CO_2 incubator. Caps should be sealed and parafilmed and placed on ice. Warm vials 2 h before using.

Preparation at the clinic prior to OPU:

1. Water bath is turned on and temperature is stabilized at 39 °C. OAP and OWP media are placed in water bath and media temperature is brought up to 39 °C prior to the beginning of OPU. Square grid dishes, 35mm plates, transfer pipets, filters, and hemostats are set-up near the stereomicroscope.

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Oocyte Aspiration Procedure

1. Aspiration is performed using OAP (oocyte aspiration plus) media. The tube is labeled with cow # and # of oocytes aspirated. The tube is allowed to sit for 10 min following aspiration in a 39 °C warming bath. The tube lid is parafilmed before placement in water bath rack.
2. A filter is placed in a 50 mL conical tube. A transfer pipet is used to remove fluid and oocytes from the bottom of conical tube, this is repeated for 2-3 more times. The filter is rinsed with OAP media to remove blood followed by OWP media. The filter is placed over a searching dish and rinsed into the searching dish with 15-20 mL of OWP media.
3. The plate is searched and oocytes are transferred into a 35 mm plate labeled with the cow #. The 35 mm contains 3 mL of OWP and is placed on the warming plate next to the scope just prior to searching the grid dish.
4. Once all the oocytes are transferred to the 35 mm plate, and washed three times in OWP, they are then transferred using a Drummond tool into the 1mL vial of SAR IVM re-parafilmed and quickly placed back into the transport incubator. Vials are placed all the way to the bottom of the incubator using a hemostat. Care must be taken to ensure vials are not dropped into the bottom upsetting the SAR IVM and oil overlay.
5. Once oocytes are transported back to the lab, loosen caps on SAR IVM vials and place into CO_2 5%, 39 °C incubator.
6. Total incubation time of 22-24hrs for bovine IVM.

Addendum:
If donors were treated with FSH, with many follicles, the follicles may be filled with blood. RLI OAP HD (OAP heavy duty) can be used to reduce the clots during OPU sessions. OAP HD is supplemented a higher concentration of heparin than regular OAP, this can significantly reduce the blood clots and possible loss of oocytes.

Fertilization *in vitro*

Preparation of Media
SAR IVF Stock II and SAR IVF III stored at -20 °C, 6 month after production date
SAR IVF Stock I stored at 4 °C, 2 month after production date
Once IVF Fertilization Medium and Sperm Washing Medium are made, the shelf life is 1 WEEK MAX!

Sperm Washing Media:

1. Add **800 µL of Stock II to 40 mL of SAR IVF Stock I** to make a total of 40mL of sperm washing medium. Label as **SAR-Sperm**.
2. Filter medium using a 0.22 µm syringe filter.
3. Aliquot (1) 1.5 mL of sperm washing per bull into a tube suitable for the size of media equilibrating, and place tubes of SAR-sperm into CO_2 incubator for 2-3 hours prior to fertilization.
   During sperm preparation 1 mL will be used to wash sperm from each bull following Isolate procedure. 0.5 mL will be added back to the 0.5 mL remaining sperm suspension following removal of supernatant.

4. Prepare a second centrifuge nipple tube with (0.5 mL) 45% and (0.5 mL) 90% Isolate layer and warm in warming bath just prior to thawing the straw of semen. Prepare one tube per bull.

Sperm washing:

1. Take semen straw from liquid nitrogen tank, gently shake in air for 10 sec.
2. Drop the straw into 37 °C water bath till straw is completely thawed, it takes about 30 sec to 1 min.
3. Cut the straw and let semen flow out straw and onto the top layer of Isolate layer.
4. Spin the centrifuge tube at 400xg for 15 min (with Eppendorf Centrifuge 5415C, it is set 2214 rpm.
5. Remove the supernatant and Isolate till the end of pellet.
6. Add SAR-Sperm 1 mL and mix by gently pipetting. Spin the tube at 400xg for 8 min.
7. Remove the supernatant and leave 0.5 mL solution in the tube, add 0.5 mL SAR-Sperm to make up 1 mL. Mix the sperm and ready for IVF.

Fertilization Media:

1. Add **770 µL of Stock III to 10 mL of stock I** to make the **fertilization media**.
2. Filter medium using a 0.22 µm syringe filter into a 15 mL conical tube and label as **SAR-Fert**.
3. To prepare for fertilization of 2 cows:
    a. Aliquot 2 mL of SAR-Fert into a 2 mL cryovial. From the cryovial pipette into a 4 well Nunc plate, 50 µL into 2 of the 4 wells. Immediately cover with 700 µL of oil.
    b. Place the remaining SAR-Fert in the cryovial into the CO_2 incubator along with the Nunc 4 well plate to equilibrate for 2-3 hours. You will need two drops of 200 µL per cow for washing matured oocytes, make in a 35 mm dish at the time of oocyte washing, take this from the remaining 2 mL aliquoted.
4. At 22-24 h of IVM, place a 60 mm dish on the warming plate and make 2 drops/cow with equilibrated SAR-Fert. Wash one cow's oocytes through 2 SAR-Fert drops (200 µL/ drop) and place into fertilization dish made before (50 µL drop of SAR-Fert covered with oil). You need to wash the oocytes very quickly.
5. Add 50 µL of purified sperm to each 50 µL drop of SAR-Fert + oocytes. Check the side of each droplet to make sure sperm is swimming vigorously.
6. Incubate dishes in CO_2 incubator at 5% CO_2, 39 °C for **exact 18 h**.

Embryo Culture with SAR IVC medium

Preparation: for 2 cows

1. Aliquot 7 mL of SAR IVC-washing medium (SAR IVC-W) into 15 mL conical tube, label IVC-W and place into CO_2 incubator at 39 °C for 2-3 h.
2. Aliquot 2 mL of SAR IVC into 2 mL cryovial, label SAR IVC, from the cryovial make a 50 µL droplet of SAR IVC in a 4 well Nunc plate for each cow and place cryovial and Nunc plate into CO_2 incubator for 2-3 h.
3. Place a 60 mm and 35 mm dish on the warming plate.

IVC Procedure:

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1. After 18 h of IVF, remove oocytes from SAR-Fert drops to a microcentrifuge nipple tube at volume of 200-300 µL and vortex for 1.5 min at the scale 7 (Fisher Scientific Vortex). Spin the vortexed oocyte tube quickly in 3-4 sec in the spinner to let oocytes spun to the bottom of tube. You need make sure that vortexing does not destroy oocytes while stripping off cumulus cells. Test your machine prior to OPU-IVF with slaughterhouse oocytes. Vortexing may lose the oocytes, spinning is important to get fertilized oocytes down to the bottom of tube.
2. Using a 200 µl pipette tip attached to a TB syringe, remove the oocytes from the bottom of the nipple tube and place in the lid of a 35 mm pre-warmed dish. Search the fertilized oocytes immediately to see if you collect all number of oocytes.
3. If not, using the equilibrated IVC-W, rinse the nipple tube with 500 µL of IVC-Wash and quickly vortex 3 to 4 times, and spin the tube quickly 3-4 sec, and remove the media from the bottom of the nipple tube and adding it to the oocyte/medium in the lid of the 35 mm dish. Continue rinsing until all oocytes are found.
   The alternative way is to denude oocytes by pipetting oocytes with glass pipets of different pore sizes. See Addendum below.
4. Using the 60 mm dish and a transfer pipette make 9 drops of IVC- Wash (at least 200 µL/drop) and 3 drops of IVC medium (at least 200 µL/drop). Wash collected oocytes through 9 drops of IVC-Wash and 3 drops of IVC medium. Then place oocytes in 50 µL drop of IVC medium. You need to wash the fertilized oocytes very quickly.
5. Culture embryos in 5% CO_2, 5% O_2, 90% N_2, 39 °C for 7 days.
6. On Day 2, check cleavage rate, must remove uncleaved oocytes from the drop. On Day 7, check blastocyst development and its rate. Uncleaved oocytes may generate toxic chemicals during the culture, and they interrupt your judgment of morulae on Day 6.

Addendum: Cumulus removal post IVF is the most important step toward to the success of IVF, for high blastocyst development of embryos.

1. You may make several glass pipets with different pore sizes, saying three-four pipets from large size to small size. The last one is just same diameter as that of oocytes.
2. Remove cumulus cells one-by-one pipet, at last time with the smallest pipet. With the largest pore size pipet, you can take several oocytes (5-6 oocytes) at a time into the pipet, and then release the oocytes. In this step, you can remove large portion of cumulus cells from embryos very quickly.
3. Change the smaller size pipets to continue removing cumulus cells, but only take 2-3 oocytes at a time since the pore size is smaller.
4. At last step, just pipet those oocytes attached with small amount cumulus cells. Do not suck whole embryo into the pipet, maybe only 1/3 to ½ of oocyte, then release oocyte immediately. By this process for two-three times, you will remove the cumulus cells completely, but not destroy the oocytes.
5. If you find the one pipet, especially the smallest pipet is fit for your removing cumulus cells easily; you can wash it in 100% ethanol several times, and save for next time to remove CC cells. This glass pipet can be your model for making next pipet, and it also can be used cumulus removal for many times.

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References

1. Senatore E, Xu J, Novoa M, Gong G, Lin T, Bella A, Moreno JF, Mannino M, Tian CX, Presicce GA, Wu S, and Du F* Improved in vitro development of OPU-derived bovine (Bos taurus) embryos by group culture with agarose-embedded helper embryos. ***Theriogenology***; 2010; 74(9):1643-1651.

2. Xu J, Guo Z, Su L, Nedambale TL, Zhang J, Schenk J, Moreno JF, Dinnyes A, Ji W, Tian XC, Yang X and Du F* Developmental potential of vitrified Holstein cattle embryos fertilized in vitro with sex-sorted sperm. ***Journal of Dairy Science*** 2006; 89:2510–2518.

3. Sung LY, Du F*, Xu J, Chang W, Nedambale TL, Jiang S, Tian XC, and Yang X The differential requirement of albumin and sodium citrate on the development of in vitro produced bovine embryos. ***Reproduction Nutrition and Development*** 2004; 44:551-564.