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# THE USE OF PERFUSED RABBIT OVARIES TO INVESTIGATE REGIONAL OVARIAN LYMPHATIC FLOW

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*ABSTRACT*

*A method is presented for the perfusion of rabbit ovaries* in vitro *which allows continuous collection of effluent perfusate from the ovarian vein and lymphatic system. The flow of ovarian lymph and the output of progesterone and 20α dihydroprogesterone in lymph and venous effluent from perfused ovaries were measured and the results compared to the same parameters measured* in vivo. *Rates of flow of lymphatic and venous effluent and lymph/plasma protein ratios measured from perfused ovaries were similar to those measured* in vivo, *and were not statistically affected by the presence of corpora lutea in the ovaries. The concentrations of progesterone and 20α dihydroprogesterone in ovarian venous blood/perfusate and lymph was increased by the presence of corpora lutea, but the concentration of progesterone was lower* in vitro *than* in vivo. *The concentration of these progestins in lymph suggest that only a small proportion of ovarian lymph is derived from corpora lutea* in vitro *or* in vivo, *and most is derived from ovarian interstitium.*

The blood vascular system of the mammalian ovary has been examined in considerable detail in several species. Rates of flow, effects of natural and pharmacological agents and stages of the reproductive cycle have been shown to exert marked effects on the types and concentrations of steroids secreted (1,2). Nonetheless, despite this extensive investigation of the ovarian blood system, considerably less is known about the function of the ovarian lymphatic system. Many have described the anatomy of this system in a variety of animals (3-10), and there have been reports dealing with the function of ovarian lymphatics in the baboon (11), cow (12), and sheep (8,10,13-18). The lymphatic vessels in these species are relatively large, which aids in their cannulation. However, the study of the function and control of the ovarian lymphatic system in common laboratory animals has been hindered by the smallness and fragility of the vessels and the resulting difficulty in collecting samples (19-21). The present report describes a method for the *in vitro* perfusion of the rabbit ovary which permits the ovarian lymphatic system and blood vascular systems to be sampled simultaneously for measurement of flow rates and concentrations of ovarian steroids in the effluents.

## *MATERIALS AND METHODS*

New Zealand White (NZW) rabbits, weighing 3 to 4kg were obtained from a local breeder and maintained on water and commercial lab chow *ad libitum*. Some rabbits received a single subcutaneous injection of 50 IU pregnant mare serum gonadotropin (PMSG) and were either sacrificed 3 days later for the *in vitro* studies or underwent laparotomy for the *in vivo* studies. Other rabbits were

injected with PMSG and three days later received an ovulatory dose of human chorionic gonadotropin (hCG, 25 IU in 0.5ml saline, intravenous); these rabbits were killed (*in vitro* studies) or laparotomized (*in vivo* studies) 8 to 10 days after the hCG.

In vivo *collection of ovarian venous blood and ovarian lymph*

Prior to the surgery, rabbits were injected (IV) with 5mg Evans Blue to help visualize the lymphatic vessels and then anesthetized (22) with intramuscular ketamine (100mg/kg) and xylazine (13mg/kg). During the laparotomy, care was taken to maintain blood pressure with the following precautions: the internal organs were wrapped with gauze sponges moistened with saline and then in cellophane; the operative site was kept moist with saline; bleeding was carefully controlled; saline was continuously delivered into the lateral ear vein via an indwelling catheter; the rabbit was situated on a heating pad to help maintain body temperature. The ovaries were positioned with the aid of Babcock forceps and the ovarian vein and lymphatic drainage located by dissecting the mesovarian fat. The vein which drains uterine blood into the ovarian vein was ligated with a single suture to insure that only blood draining from the ovary was collected. Although several lymphatic vessels drain the ovary, they join into a single lymphatic duct near the midline. This duct was located with the aid of the Evans Blue color, 5000 IU heparin was injected IV and the vessel was cannulated with PE10 tubing pulled into a smaller tip and secured with suture. Lymph was collected in a tube placed in an ice bath and the flow rate determined by timing the individual drops as they fell from the cannula tip. Once an adequate sample of lymph had been collected, the lymphatic cannula was removed and the ovarian vein was cannulated with PE90 tubing; about 4ml of venous blood was collected. At the end of the procedure, the rabbit was sacrificed by lethal injection of euthanasia (Parke-Davis) containing pentobarbital and methanol.

In vitro *collection of ovarian venous and lymphatic effluent in perfused ovaries*

Immediately prior to sacrifice, each rabbit received 5000 IU heparin and a peripheral blood sample was collected. The rabbits were killed by a lethal injection and the abdominal cavity was quickly opened and the mesovarium freed of any attachments to the lateral abdominal wall. By gently pulling on the mesovarium, the ovary and surrounding tissue were spread over the plastic slotted frame of a 3.5 inch plastic embroidery ring and the tissue secured with the spring loaded metal retaining ring. This maneuver helped maintain the spatial relationships of the vessels and the ovary and also ligated the vessels when the tissue was cut free from the rabbit. The ovaries were transported to the laboratory submerged in ice cold Krebs Ringer bicarbonate buffer (pH 7.2). The ovary in its retaining ring was placed in a shallow glass dish containing Krebs Ringer bicarbonate buffer which sat atop a platen designed for slab gel electrophoresis and attached to a circulating water bath. The platen temperature could be controlled so that the bath medium temperature was regulated between 4°C and 34°C; the medium was continuously gassed with 95% $O_2$ and 5% $CO_2$.

The ovarian vessels were located in the periovarian fat and the ovarian artery cannulated with PE50 tubing pulled to a tip of outside diameter (OD) of 0.51 mm and inside diameter (ID) of 0.36 mm. The ovarian vein was cannulated with PE90 tubing with OD in the range of 0.9 to 0.6 mm and ID in the range of 0.7 to 0.45 mm. The maximum allowable cannula (based on the diameter of the ovarian vein) was inserted. Branches of these vessels which were not involved with the ovarian blood flow were identified and ligated with microclips. Once the cannulae had been secured with sutures, the oxygenator and pump assembly depicted in *Fig. 1* was turned on to begin perfu-

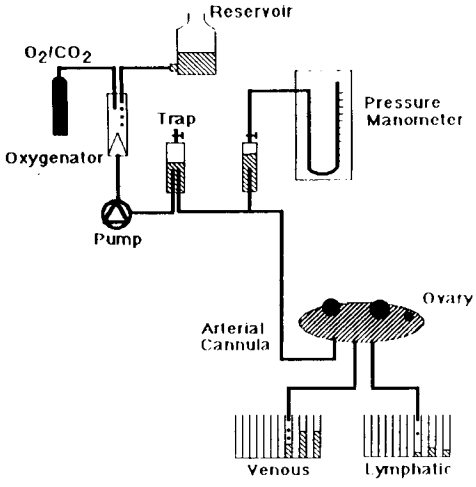


*Fig. 1. Diagrammatic representation of the procedure used to perfuse the rabbit ovaries and collect venous and lymphatic outflow. The pump speed was set for a perfusion pressure of 50mmHg. Venous and lymphatic effluents were collected in separate fraction collectors.*

sion. The perfusion medium was Krebs Ringer bicarbonate buffer with 4% bovine serum albumin (BSA) and 0.02 IU heparin/ml, 0.2 IU insulin/ml and 0.94mM glucose which was gassed continuously with 95% $O_2$:5% $CO_2$ mixture. Venous flow was adjusted to approximately 1ml/min at a perfusion pressure of 50mmHg with the venous pressure set at 0mmHg. The partial pressure of oxygen in the perfusate in the arterial cannula was generally in the range of 400-500mmHg with carbon dioxide values of 30-35mmHg and a pH of 7.4. Although the initial dissections of the ovarian vasculature were carried out with a bath temperature of 4-10°C, once the cannulae were in place, the temperature of the bathing medium was raised to 34°C.

Once the arterial and venous cannulae had been positioned and secured and the ovary cleared of blood, the pedicle site was closely examined under 20X magnification to locate the ovarian lymphatic duct. In some instances, the clear, minute lymph vessel was visible on the surface of the fat while at other times, it was necessary to add to the perfusion buffer Evans Blue, a dye which was transferred into the lymphatic drainage in small amounts rendering the transparent lymphatic vessels pale blue while blood vessels were stained darkly blue. The main lymphatic duct was cannulated with PE 10 tubing which had been pulled into a tip of OD 0.23 and ID 0.11 mm and secured with sutures. During the perfusions, lymphatic outflow pressure was maintained at -3 mmHg. Both the venous and lymphatic cannulae drained into fraction collectors containing preweighed tubes and by timing the fraction collectors, the venous and lymphatic flow rates could be calculated.

*Determination of progesterone, 20α dihydroprogesterone and protein content of venous and lymphatic effluent*

Steroid content of the venous plasma and lymph (*in vivo* studies) and venous and lymphatic effluents (*in vitro* studies) were determined by the method of Mills and Osteen (23) modified to take into account the small volumes of ovarian lymphatic effluent collected. The protein content of serum, lymph, and perfusion medium was determined by the method of Lowry et al (24) as modified by Ogle (25).

## *RESULTS*

At a perfusion pressure of 50mmHg, the flow from the ovarian vein of the perfused ovaries was 2.2±0.34ml/min/gm tissue while the lymphatic effluent flow rate was 23.7±5.3μl/min/gm. The presence or absence of corpora lutea did not have a statistically significant effect on either venous or lymphatic flow rates.

*Fig. 2* presents a comparison of the *in situ* ovary and the perfused ovary with and without corpora lutea in terms of the lymphatic and venous content of progesterone and 20α dihydroprogesterone. It is clear that in both systems, the *patterns* of secretion are nearly identical, but that the perfused ovaries produce less steroid than the *in situ* ovaries. It should also be noted that in both systems, the presence of

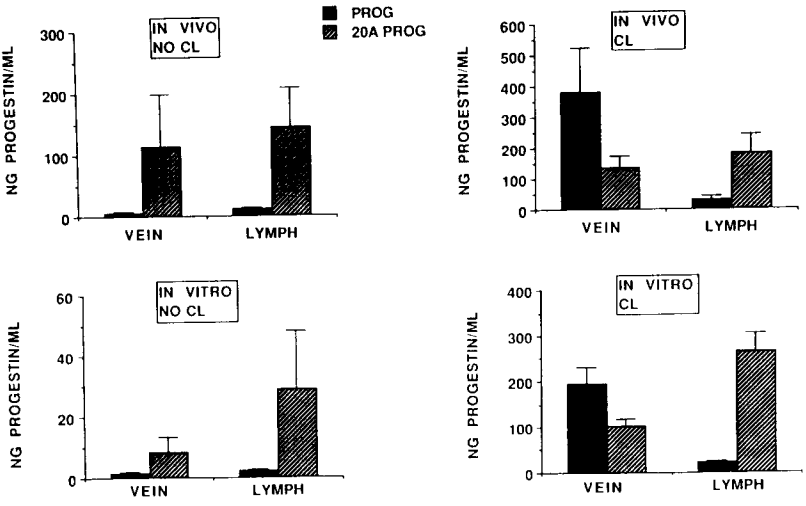


*Fig. 2. The secretion of progesterone (PROG) and 20α dihydroprogesterone (20A PROG) by* in situ *ovaries (*IN VIVO*) and perfused ovaries (*IN VITRO*) with and without corpora lutea (CL). Each bar represents the mean ± SEM of 11 to 16 ovaries (*IN VITRO *studies) or 4 to 6 ovaries (*IN VIVO *studies).*

luteal tissue elevates progesterone in the ovarian venous effluent by 20 to 40 fold while the concentration of this progestin in the lymphatic output rises only 2 to 3 times. This pattern is different from that of 20α dihydroprogesterone where the metabolite is present in greater concentrations in the lymphatic effluent than in venous outflow.

The concentration of protein in the effluent collected from the venous and lymphatic vessels *in vivo* and during *in vitro* perfusion with buffer containing 4% BSA is shown in *Fig. 3.* In both systems, the lymphatic effluent draining ovaries with and without corpora lutea contains about 90-95% of the protein present in the venous effluent from the same ovaries. These measurements point to the extreme leakiness of the ovarian vasculature which permits the loss of large amounts of plasma proteins into the interstitial space and eventually into the lymph.

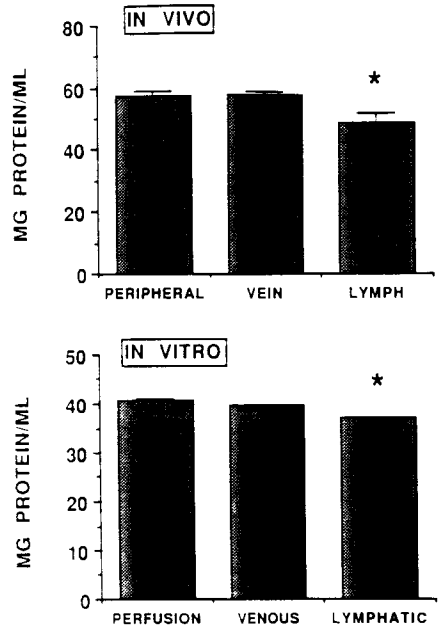


*Fig. 3. The protein content of peripheral plasma, ovarian vein plasma and ovarian lymph (*IN VIVO *studies) and perfusion medium, ovarian venous effluent and lymphatic effluent (*IN VITRO *studies). Each bar represents the mean ± SEM of 7 to 10 determinations. * Indicates that LYMPHATIC is less than PERIPHERAL and VEIN (*IN VIVO *experiments) or less than PERFUSION and VENOUS (*IN VITRO *experiments).*

## *DISCUSSION*

Much of what is known about the ovarian lymphatic system comes from the study of the sheep ovary when corpora lutea are present and the lymph vessels

are large and can be readily cannulated. Characteristically, in these studies, the ovarian lymphatics are cannulated with polyvinyl chloride tubing and the cannulae are exteriorized to drain into a collection bottle attached to the animal (8,10,12-18). These procedures generally permit the collection of lymph from luteal phase ovaries over a period of several hours or even days. Investigators report flow rates of 3-8ml/hr and that the lymph contains high concentrations of progestational steroids and protein concentrations of 70-90% of that in the ovarian venous plasma. Bovine luteal phase ovarian plasma has also been collected using similar methods (12). In the cow ovary, average flow rates of 22.5ml/hr are reported along with evidence of rhythmic contractions of the lymphatic vessels. For the bovine studies, a specialized cannula is used that permits the continuous infusion of an anticoagulant to prevent clotting in the cannula and to allow long term collection of ovarian lymph.

The collection of ovarian lymph from smaller laboratory animals has apparently been less successful. Dharmarajan et al (19) inserted a sharp polyethylene cannula into the ovarian lymphatic drainage of 16 day pregnant rats injected with Patent Blue. These investigators reported an average ovarian lymph flow rate of 1.1$\mu$l/min containing progesterone and 20$\alpha$ dihydroprogesterone and a lymph/plasma protein ratio of 0.53. We have previously cannulated the ovarian lymphatic system of the rabbit ovary and compared the progesterone and 20$\alpha$ dihydroprogesterone content of lymph to ovarian vein plasma levels collected from the same ovary (20,21). Rabbit ovarian lymph showed only small increases in the levels of progesterone when corpora lutea were present while the rise in venous progesterone was great. These results, along with the rat studies of Dharmarajan et al (19) seem to be in disagreement with other reports which showed that the progesterone content of ovine (13) and bovine (12) ovarian lymph is very high when corpora lutea are present. An explanation of the disparity is found in the fact that the larger animals demonstrate lymphatic vessels within the corpora lutea, but neither the rat nor the rabbit corpus luteum contains lymphatic spaces except in the peripheral portions derived from the follicular thecal layer (26,27). Ichikawa et al (27) have suggested that lymphatic vessels are not developed within the rapidly growing rabbit corpora lutea because extravasated or secreted interstitial material readily diffuses to the medulla where an extensive lymphatic network already exists. The present studies support the histological observation of no luteal lymphatics because steroid analysis indicates that in the rabbit, secretions from the corpora lutea are removed via the venous system. On the other hand, the primarily medullary interstitial products are transported in the lymph. In large species (cow, sheep), however, an extensive lymphatic drainage system develops in the more slowly growing corpora lutea and results in very high lymphatic concentrations of progesterone.

In the present report, we describe the use of a perfused rabbit ovary system to study the flow rates and steroid content of ovarian lymph. In the studies of the actions of a variety of agents on ovulation and steroid production, perfused rabbit ovaries have been previously used by two important research groups. In experiments described by Dharmarajan et al, rabbit ovaries were perfused with Medium 199 containing antibiotics and anticoagulants (28); these investigators also reported that the addition of 3% BSA to the perfusion medium reduced the edema in the tissues. This group generally used a perfusion pressure that led to a venous flow rate of 1-2ml/min. The other group that perfused rabbit ovaries [Ahren and Janson and coworkers (29,30)] used Medium 199 with 4% BSA, anticoagulants, antibiotics and perfused in a recirculating system at an arterial pressure of 30-40mmHg. In the present study, we used Krebs-Ringer bicarbonate buffer supplemented with heparin, insulin, glucose, and 4% BSA with a perfusion pressure of 50mmHg. However, the actual perfusion pressure within the ovarian

artery was likely less than 50mmHg due to the reduction in the diameter of the tip of the cannula through which the perfusion buffer had to flow. When the cannula tip was pulled out from an ID of .58mm to .36mm, the reduction in the cross-sectional area was greater than 50%. The resultant added resistance to flow effectively reduced thereby the pressure at the cannula tip.

In our system, the *in vitro* arterial perfusion pressure of 50mmHg resulted in ovarian venous flow rates of 1-3ml/min/gm ovary and ovarian lymph flow rates in the range of 10-50$\mu$l/min/gm ovary. The venous flow rate range compares favorably with several prior reports of *in vivo* rabbit ovarian blood flow using radioactive microspheres which generally fall into the range of 2-4ml/min/gm (1,2). Measurement of the *in vivo* ovarian lymph flow rate is made difficult by the problems of maintaining the position of the cannula and the difficulty in locating and ligating all of the non-ovarian lymph drainage. Nevertheless, in several instances, we measured lymph flow rates in the range of 5 to 20$\mu$l/min/gm.

Ideally, the perfused ovary should exactly mimic the *in vivo* ovary in terms of blood and lymph flow rates and steroid concentrations. In several respects, we have been successful with the perfused ovary model described herein. For example, the pattern of progesterone and 20$\alpha$ dihydroprogesterone in the venous and lymphatic effluent of the perfused ovary is nearly the same as the pattern of these steroids in the ovarian venous plasma and ovarian lymph collected *in vivo*. Similarly, the difference in protein content of the venous and lymphatic effluent is nearly the same as the *in vivo* difference (21). Despite the similarities, however, there is an important difference in the two systems: the concentration of progesterone in both the *in vitro* venous and lymphatic effluents is significantly less than the concentration measured *in vivo*. This difference could be due to the absence of steroid substrate and/or steroid binding proteins in the perfusion buffer or due to an experimental artifact such as the perfusion temperature being lower than normal body temperature, or, alternatively, possible damage to the blood vasculature during operative preparation of the ovary for perfusion. The isolated ovaries are also transported to the laboratory and stored for various periods of time in ice cold Krebs-Ringer bicarbonate buffer which is gassed with oxygen/carbon dioxide immediately before use. The period of cold ischemia (period between removal of the ovaries from the rabbit and the start of perfusion with oxygenated buffer) was usually about 30 minutes for the first ovary although it was up to 3-4 hours for the second ovary from the same rabbit. Accordingly, there may have been some loss in the responsiveness of the blood vessels to vasoactive agents during this more prolonged period.

A distinct advantage of ovarian perfusion is that it permits a level of control over the blood and lymph vasculature which is not possible in the intact rabbit. By removing the ovary and associated vasculature from the rabbit, the actions of pharmacological agents and alterations in arterial perfusion pressure and lymphatic outflow pressure can be isolated without the complications introduced by the actions of these agents and procedures on the systemic circulatory system. Furthermore, by using high magnification and Evans Blue to examine the periovarian tissues, it is possible to identify and ligate regional blood and lymphatic vessels from the oviduct, uterus and surrounding fat which could dilute the samples with non-ovarian blood or lymph. The use of the embroidery ring to position the ovary during the cannulations also minimizes the movement of the ovary induced by the rhythmic contractions of the mesovarium and other mesenteries. *In vivo*, these mesenteric contractions lead to considerable ovarian motion and often cause the lymphatic cannula to change position with disruption in lymph flow. Finally, using the perfused ovary, arterial perfusion pressure can be kept constant for extended periods of time without the problem of slowly declining blood pressure and accompanying fall in lymph flow which

often occurs with lengthy surgical procedures.

Although there are some drawbacks to the use of the perfused rabbit ovary as a model system for the study of the ovarian lymphatics, we suggest that this experimental preparation is useful for preliminary studies of factors which may alter the flow or composition of ovarian lymph and thereby help define the role of the lymphatic system in ovarian function (31,32).

## *ACKNOWLEDGEMENTS*

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