ABSTRACT

A representative blood sample from the mammary vein depends on the functional integrity of the valves in the external pudic vein (EPV). To determine if the EPV valves maintain blood flow into the inguinal direction during the second and subsequent lactations, we used eight lactating cows catheterized in the EPV, the lateral branch of the cranial mammary vein (MV), and the external pudic artery (EPA). The averaged daily milk yields were 25.0 ± 1.8 kg in cows in second lactation and 31.5 ± 2.9 kg in older cows. The relative time taken by a pulse dose of p-amino hippuric acid (PAH) injected into the EPV, to reach the EPA and the MV, was measured in a first trial. In a second trial, we assessed the extent of alteration of the mammary PAH blood concentration with blood originating from other tissues using a continuous infusion of PAH into the EPA simultaneously with blocking or not any EPV backflux. From the first experiment, the PAH injected into the EPV appeared first in the EPA and then in the MV in cows in second lactation, suggesting that blood flow was towards the inguinal region. But in a third-lactation cow, the order of appearance was reversed. In parallel, the occlusion trial demonstrated that the concentration of PAH in the MV was diluted by 14 to 39% with blood draining nonmammary tissues only in cows in third or fourth lactation. This resulting reversed flow from the EPV towards the MV would have a detrimental impact on conclusions of mammary gland metabolism studies conducted with cows in their third lactation or higher.

INTRODUCTION

The characteristic distensibility of the vein wall allows the venous circulation to develop adaptive mechanisms in accordance with hemodynamic alterations. Valves of the venous system become nonfunctional or sometimes are even destroyed when veins are overstretched by excessive blood pressure. The loss of functional integrity occurs when the vein cross-sectional area increases and the leaflets of valves cannot close completely (12).

Linzell (15) reported valvular incompetency in the main veins draining the mammary glands in lactating ruminants. These veins exhibited all the characteristics of varicose veins. He also suggested that a similar situation could occur in other species having inguinal mammary glands (17). Following the increased blood supply required to support mammary tissue development and milk synthesis, the external pudic vein (EPV) wall is stretched, provoking a loss of the effectiveness of its valves. This loss of functional integrity would imply a backflux from the caudal epigastric and the femoral veins into the lateral branch of the cranial mammary vein (MV; 2). Contamination of the MV blood with venous blood from other tissues would bias measurements of arteriovenous concentration difference and flux across the mammary gland, as also discussed by Bequette et al. (3).

We designed two experiments to determine, in relation to lactation number of lactating cows, 1) the mammary venous pathway of blood flow and 2) the magnitude of the alteration of mammary venous concentration of p-amino-hippuric acid (PAH) infused in the external pudic artery (EPA), which would be induced by a loss of functionality of the EPV valves.
MATERIALS AND METHODS

Animal Care and Surgical Procedures

Two separate experiments were conducted on two groups of four lactating Holstein cows. Cows were maintained in tie stalls equipped with rubber mats and bedded with straw. They were individually fed ad libitum, according to NRC recommendations (20) and had free access to water. Experimental procedures were approved by a local animal care committee and conducted in respect of recommendations of the Canadian Council on Animal Care (4). Indwelling catheters were placed surgically in the left EPA, the left EPV, and in the left MV in cows used in the first experiment (Figure 1). For the purpose of the second experiment, indwelling catheters were placed in the left EPA and the left MV (Figure 1). A transit-time ultrasonic flow probe was placed around the left EPA of all cows. Surgical procedures were conducted approximately 4 wk before the beginning of experiments.

Materials. Arterial and venous sampling Tygon catheters used were: 1.0 mm i.d. and 1.8 mm o.d. for the arterial catheter; 1.3 mm i.d. and 2.3 mm o.d. for the venous catheter (Norton Performance Plastics, Akron, OH). The model of transit-time ultrasonic flow probe that was used was: model 12S-L reflector (Transonic Systems Inc., Ithaca, NY). The occlusion Fogarty catheter model used was: inflated balloon maximum diameter: 45 mm, length: 80 cm, model number: 62080822F (Baxter Corporation; Edwards Division, Mississauga, ON, Canada).

Blood flow probe and sampling catheters. Forty-five minutes before the surgery, cows were sedated with acepromazine maleate (10 mg/100 kg; Atravet, Ayerst Laboratories, division of Wyeth-Ayerst, Montréal, QC, Canada). Anesthesia was induced with 0.8% thiopental sodium (Pentothal, Abbott Laboratories, Montréal, QC, Canada) and 5% glycerol guaicolate solution (Denis Giroux Laboratories, QC, Canada) given i.v. at 1 ml/kg of BW or until anesthesia was reached. Anesthesia was maintained with 2 to 3% isoflurane (Isofo, Solvay Animal Heath Inc., Kitchener, ON, Canada) and 6 L of O2/min. A 15- to 17-cm incision was made over the inguinal region under aseptic conditions, between the medial thigh and lateral surface of the mammary gland. A purse string suture was placed on the wall of the left EPA for the insertion of 17 cm of the catheter. The left EPV catheter was placed by the same procedure, where 10 cm of the catheter was inserted. An ultrasonic blood flow probe was placed around the left EPA of all cows. Surgical procedures were conducted approximately 4 wk before the beginning of experiments.

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Occlusion Fogarty catheter. Approximately 15 min before the surgery, each cow was sedated with Atravet (10 mg/100 kg) intramuscularly. Local anesthesia was performed by infiltrating a transverse line of 2% hydrochloride lidocaine (Xylocaine, Astra Pharma Inc., Mississauga, ON, Canada). A cutaneous incision of 2 cm was made over the caudal superficial epigastric vein and a purse string suture on the vein wall encircled the vein incision for the Fogarty catheter insertion. The catheter was inserted so that the balloon was in the lateral branch of the mammary vein, cranially to the anastomosis with the EPV. This

![Figure 1. Mammary vascularization. Legend; 1: the femoral vein; 2: the caudal epigastric vein; 3: the external pudic artery; 4: the external pudic vein; 5: the lateral branch of the cranial mammary vein; 6: the caudal superficial epigastric vein; 7: the caudal mammary vein; 8: the perineal vein; 9: the perineal artery; 10: the occlusion site; 11: the sampling site; 12: ultrasonic blood flow probe. Stippled vessels indicate arteries and clear vessels indicate vein. Schema adapted from Gorewit et al. (9).](image-url)
measurement was individually determined during the previous surgery. An adhesive elastic bandage was placed over the exterior part of occlusion catheter, along the abdomen. The complete surgery required approximately 20 min and was performed the day before the experimentation.

**Trials**

**Experiment 1a. Pulse dose injection of PAH into the EPV.** Four cows were used, two in the second lactation, one in the third and one in the fourth lactation. Respective daily milk yield and BW averaged 25.3 ± 2.1 kg and 647 ± 17 kg in cows in second lactation, and 33.1 ± 1.4 kg and 609 ± 113 kg in older cows. Respective DIM were: 251 and 234 for cows in the second lactation, 79 for the cow in the third lactation, and 85 for the cow in the fourth lactation. To determine the direction of the external pudic venous flow, a 15-ml pulse dose of PAH (10% wt/vol) was injected into the EPV. Starting at the beginning of the injection, blood samples were taken simultaneously from both the EPA and the MV every 10 s during the first 2 min and every 20 s during the following 2 min. The sampling procedure allowed the required time to a first return of PAH into the EPV to be determined in relation to the appearance of PAH in the mammary venous blood. The time required for tracer appearance in the MV would be longer if the blood in the EPV flowed into the inguinal direction rather than in the MV. In the former case, the tracer would need to be mixed in the general venous blood before returning to the mammary gland by the arterial blood and would then appear in the MV. Unfortunately, difficulties with catheter patency restricted the number of observations. When PAH was injected into the EPV, a cow in second lactation could not be sampled into the EPA, and the fourth-lactation cow could not be injected with PAH due to a nonpatent EPV catheter. The animals were standing and blood flow of the EPA was recorded continuously during the experiment.

**Experiment 1b. Pulse dose injection of PAH into the EPA.** A pulse dose of 15 ml of PAH was injected into the EPA with the same cows, procedures, and blood sampling protocol as in the experiment 1a. Blood was simultaneously sampled from both the EPV and the MV, giving the respective time of appearance of PAH into the main mammary veins. Difficulties with the patency of the EPV catheter reduced the number of observations; only the third-lactation cow could be sampled into the EPV. The animals were standing and blood flow of the EPA was recorded continuously during the experiment.

**Experiment 2. Occlusion trial.** Four additional cows were used, two in the second lactation, one in the third, and one in the fourth lactation. Respective daily milk yield and BW averaged 24.8 ± 2.1 kg and 669 ± 36 kg in cows in second lactation, and 30.2 ± 2.6 kg and 698 ± 13 kg in older cows. Respective DIM were 228 and 216 for cows in the second lactation, 128 for the cow in the third lactation, and 146 for the cow in the fourth lactation. The extent of alteration of the MV PAH concentration with blood from nonmammary origin was assessed with a continuous PAH infusion into the EPA, which is the main mammary arterial supply, and an occlusion Fogarty catheter was placed in the MV, cranial to the anastomosis with the EPV. Occlusion of the MV allowed us to determine the dilution of the PAH concentration in the MV blood by the EPV blood backflux, which would include blood coming from the femoral and the caudal epigastric veins. The concentration of PAH in blood of the two latter veins would be characterized by a more diluted PAH concentration than the blood coming out directly from the mammary gland. Consecutively to the infusion of the tracer into the arterial supply of the mammary gland, any venous blood coming out from other tissues would dilute the infused PAH. Obstruction and nonobstruction treatments were assigned over three 30-min periods, according to a switchback design. A continuous infusion of PAH (2.4 ml/min; 10% wt/vol) was performed into the EPA, beginning 45 min before the onset of measurements and was preceded by a priming dose injection (15 ml; 10% wt/vol). Blood samples were withdrawn from the MV every 3 min during the last 15 min of each period. They were analyzed individually for PAH concentration and the five values were averaged over each period, making one concentration of PAH per period for statistical comparison of mean effect of treatments. Fifteen minutes was allowed between the obstruction or nonobstruction of the site and blood sampling to ensure that the physiology of the venous circulation was not disturbed by the distension caused by balloon inflation. The patency of catheters was verified before the experiment. The animals were standing during overall period of measurement. Blood flow of the EPA was recorded continuously during the experiment and was averaged over each of the three 15-min periods for statistical comparison of the mean effect of treatments.

**Laboratory and Statistical Analyses**

Blood samples were collected in heparinized Vacutainers, and kept on ice. Right after sampling, they were centrifuged at 2000 x g for 10 min at 4°C and stored frozen at −20°C until further analysis. Plasma
PAH concentration was analyzed within 14 d after sampling with an automatic analyzer (Technicon Autoanalyser II, Technicon Instruments Corporation, Tarrytown, NY), according to Huntington (13).

Mean effects of obstruction or nonobstruction of any backflux from the EPV into the MV on the PAH concentration in the MV and on the averaged arterial blood flow, were analyzed using general linear models procedures of SAS (23) as a switchback design involving three periods. The following independent variables were used in the model with their respective degrees of freedom given in parentheses:

\[ Y_{ijkl} = \mu + \text{lact}_i + \text{cow(lact)}_{j(i)} + \text{per}_k + \text{trt}_l + \text{trt}_l \times \text{lact}_i + e_{ijkl}, \]

where

- \( Y_{ijkl} \) = dependent variable,
- \( \mu \) = overall mean,
- \( \text{lact}_i \) = effect of lactation number \( i \), (1 df): second lactation or greater than 2nd lactation
- \( \text{cow(lact)}_{j(i)} \) = effect of individual cow \( j \) nested within lactation \( i \), (2 df)
- \( \text{per}_k \) = effect of period \( k \), (2 df)
- \( \text{trt}_l \) = effect of treatment \( l \), (1 df)
- \( \text{trt}_l \times \text{lact}_i \) = interaction between treatment \( l \) and lactation \( i \) variables, (1 df) and
- \( e_{ijkl} \) = residual error associated with the following observations \( ijkl \), (4 df).

The period within lactation effect was not significant (\( P > 0.20 \)) and was pooled in the error (14).

**RESULTS AND DISCUSSION**

**Experiment 1a. Pulse dose injection of PAH into the EPV.** The PAH injected into the EPV of the third-lactation cow resulted in an increment of PAH concentration in the MV 20 s after the beginning of the injection (Figure 2a). The increment of PAH concentration in the MV of the cows in second lactation was measured 40 to 50 s after the onset of the injection. The maximum PAH concentration in the MV was reached approximately 10 to 30 s after the appearance of PAH at the sampling site. The injection of the bolus, which lasted 25 to 30 s, was apt to flatten out the maximum concentration of the downstream peak of PAH. But the 10- to 30-s delay to measure the maximum PAH concentration was likely a result of laminar flow in large blood vessels and a range of different length pathways that a bolus must traverse from injection to sampling site (19). In the cow in third lactation, the PAH appearance into the external pudic arterial blood occurred 10 to 20 s later than into the MV (Figure 2b). In contrast, in the cow in second lactation, PAH appeared first into the EPA, 20 s after the onset.
of the injection, and was then followed by an appearance into the MV 20 s later (Figure 2c). In both cows in second and third lactations, the maximum PAH concentration measured in the EPA was reached 20 s after the first appearance of PAH into the EPA. According to the anatomy of the mammary vascular system of the cow (Figure 1), PAH injected into the EPV should be drained into the inguinal direction. With functional valves in the EPV, PAH injected into the EPV should flow through the inferior vena cava, into the right atrium of the heart, through the lung oxygenation pathway, and then into the arterial circulation including the mammary supply (2). This theoretical flow agrees with observations made in the cows in second lactation. Although the arterial blood was obtained only from one of the two cows in second lactation, the time of appearance of PAH into the MV was similar for both cows. This suggests that PAH appeared first in arterial blood and then in the mammary venous blood after its administration into the EPV. Consequently, the dilution of PAH concentration measured in the MV of the cows in second lactation resulted from a dilution of the pulse dose with general circulation, being prerequisite to a first return at sampling site. In contrast, PAH injection into the EPV of the cow in third lactation appeared to be drained directly into the cranial direction into the MV, as indicated by a first appearance and a very high PAH concentration in the MV, and then followed by the appearance in the EPA. This implies nonfunctional valves, allowing a backflux from the EPV into the MV only observed in the cow in third lactation but not in the cows in second lactation used in this study.

**Experiment 1b. Pulse dose injection of PAH into the EPA.** Injection of PAH directly into the EPA resulted in PAH appearance in the MV 10 to 20 s after the onset of the injection and a maximum concentration was reached 20 to 30 s after the appearance of PAH into the MV, with no difference between lactations (Figure 3a). The complete injection of PAH into the EPA required 27 to 30 s. A similar PAH transit time across the mammary tissue has been observed when the injection was performed into the EPV of the cow in second lactation (Figure 2c). The high resistance to flow in capillaries imposes low blood velocity, averaging 0.1 to 1 mm/s, depending on tissue studied (11). The resulting transit time across the mammary glands integrates the sum of the respective transit times across mammary arteries, capillaries, and veins (8). Simultaneous blood sampling from both the MV and the EPV, while the PAH was injected into the EPA, demonstrated that the pulse dose was predominately drained into the MV in the third lactation cow (Figure 3b). These results agree with those for the same third-lactation cow in experiment 1a. The MV increment of PAH concentration was measured 20 s after the onset of the injection and the maximum concentration was reached 20 s after its appearance. The PAH appearance in the EPV exhibited a lag time when compared to the time of appearance into the MV. An increment of concentration of PAH in the EPV was measured 70 s after the onset of the injection with a maximum concentration reached 10 s later. This return of PAH into the EPV is expected to come from the femoral and the caudal epigastric veins. Therefore, the pulse dose injected into the EPA would be
submitted to a first return to the heart and then followed by arterial perfusion of tissues drained by the femoral and epigastric veins. Then, the total transit time was relative to the vasculature of the perfused tissues (7). The regularity of PAH transit-time measured across the mammary tissue ensured that procedures were consistently performed among animals and those two protocols.

Experiment 2. The occlusion trial. While a continuous infusion of PAH into the EPA was carried out, the occlusion of the MV, cranially to the anastomosis with the external pudic vein, during continuous infusion of p-amino hippuric acid (PAH; 10%) into the external pudic artery: variation of PAH concentration in the MV. Dotted areas indicate occlusion period. Legend: ○○○○○○○ and ■—■—■ indicate cows in second lactation; ——— and ——■—■—■ indicate cows in third and fourth lactation, respectively.

![Figure 4. Effect of the occlusion of the lateral root of the cranial mammary vein (MV), cranially to the anastomosis with the external pudic vein, during continuous infusion of p-amino hippuric acid (PAH; 10%) into the external pudic artery: variation of PAH concentration in the MV. Dotted areas indicate occlusion period. Legend: ○○○○○○○ and ■—■—■ indicate cows in second lactation; ——— and ——■—■—■ indicate cows in third and fourth lactation, respectively.](image)

The PAH concentration was similar between periods 1 and 3 of the three consecutive periods (Figure 4). Similar PAH concentrations in periods 1 and 3, when the same treatment was applied as a result of
the switchback design, would indicate that inflation or deflation treatments did not disturb the normal physiology of venous circulation (Figure 4). Arterial blood flow in the left mammary gland was similar between lactation number and averaged 3.00 and 2.90 L/min (SE 0.076; \( P = 0.38 \)) in second lactation or greater, respectively. Furthermore, the inflation of the balloon did not alter the left external pudic arterial blood flow when compared with unobstructed period (2.89 and 3.01 \( \pm 0.078 \) L/min; \( P = 0.35 \)). In other studies, lower blood flow in the MV or in the EPA had been reported when a manual clamp of the EPV was applied in dairy goats (16, 21).

An increase in the required nutrients, as a result of an increase in tissue metabolism, is related to a decrease in resistance of the tissue capillaries, thus exposing cells to higher flow of nutrients (5). The resulting supply of venous blood exerts a pressure on the wall of venules and veins that subsequently readjust their muscular tension to return to their initial pressure. Therefore, the veins that drain the udder would become distended when they are overstretched by excess of venous pressure lasting weeks or months, as stated in the general concept of physiology of the venous circulation (12). Linzell (15) has reported this physiological adaptation in relation to milk production and he observed that those veins had all the characteristics of varicose veins. It is the mechanism of venous compliance that allows maintenance of low venous pressure by increasing the vein diameter when an extra volume of blood must be drained, although large veins are less compliant than smaller veins (6, 12). Varicose veins are the result of maintaining over a long period a disproportion between the venous pressure and the resistance of the wall of the vein (1, 10).

The pathologic changes in varicose veins are as follow: 1) increase in the diameter; 2) elongation and tortuosity; 3) loss of elasticity from increase in fibrous connective tissue; 4) variations in the thickness of the wall; and 5) disappearance or atrophy of valves (1). In the absence of the latter event, the leaflets of the valves do not increase in size, such that an increase of the vein cross-sectional area over a certain limit induces a loss of valvular functional effectiveness (12). Fluids flow in the direction of lower resistance; consequently, blood of the EPV flows into the MV, rather than toward the inferior vena cava when EPV valves become non-functional.

An overview of the hemodynamic alteration of the mammary tissue indicates that the mammary venous circulation adapts to the milk synthesis potential. The relative rate of venous flow to high milk synthesis, maintained over long period, would provoke a gradual loss of integrity of the vein wall and the functional integrity of the EPV valves. The progressive development of incompetency of individual venous valves results in a chain reaction. Once one or more valves become incompetent, due to dilatation of the vein wall, the eventual retrograde flow will increase hydrostatic pressure on successive valves and wall portion, which themselves dilate and the development progresses downward, and not only in the main channel but also in tributary veins (10). Once the vein loses its elasticity from an increase in fibrous connective tissue as in varicose vein, the damage caused is chronic (22). In the current study, two third- and one fourth-lactation cows demonstrated a reversed flow in the EPV. Among cows in second lactation studied, neither showed a reversed blood flow into the EPV.

The wall of the EPV and the MV are submitted to different pressures during a whole lactation. The veins and the valves have the physiological capacity to support an increase in venous pressure over a certain period. Healthy valves resist to 250 mm Hg of pressure, while when they become incompetent, backflow occurs at 10 to 40 mm Hg in dairy goats (15). Linzell (15) stated that the EPV or the MV exhibiting reversed flow had the characteristic of varicose veins. But, the physiological change toward reversed flow would appear to be consecutive to a gradual loss of individual valve competency in goats and cows (15). In humans, it has been suggested that incompetency of the valves in varicose veins is actually secondary to their dilatation (1).

In the occlusion trial, arterial blood flow of the left mammary gland was not different between cows in second lactation (3.00 L/min) and in third lactation and greater (2.90 L/min; \( P = 0.38 \)). So, even if cows in second lactation had more DIM (222 d) than cows in their third and fourth lactations (137 d), the pressure exerted on the venous wall of the EPV would be similar among these cows. The valves in the EPV were still able to withstand the pressure exerted by the venous flow in those two cows in second lactation. Similar arguments could be stated regarding cows used in the protocols of PAH injections into the EPV and EPA. Cows in second lactation had more DIM (242 d) than the cow in the third lactation (79 d). Respective blood flow in the left mammary gland averaged over the period of measurement were 4.19 and 3.65 ml/min in cows in second lactation and 3.49 L/min in the cow in third lactation, no statistical comparison could be performed on those data. Linzell (15) reported that the EPV valves maintained blood flow into the inguinal direction during the first lactation. Our results show that reversed flow in the EPV appears in third- and fourth-lactation cows, but given the small number of animals used and individual variation in milk produc-
tion and in vein structure (1), we cannot dismiss its possible earlier appearance in individual cows.

Valves in both the cranial mammary vein and the superficial epigastric vein have been reported to become nonfunctional at first pregnancy (15, 18). In virgin females, the cranial mammary vein blood flows towards the inguinal direction, in the same direction as the valves (15). From first pregnancy and in lactating ruminants, the increased mammary blood flow leading to an increase in vein diameter and nonfunctional valves caused the blood to flow into the mammary vein in cranial direction, against the valves (15, 17). Such a situation implies a return of the perineal vein blood, draining the anus and vulvae, into the cranial mammary vein. However, its influence on how representative the mammary venous blood sample was has been reported to be negligible (17).

To overcome consequences of the EPV reversed flow in studies necessitating cows in third lactation and higher, the middle branch of the cranial mammary vein could be alternatively sampled. Its anastomoses with the subcutaneous abdominal vein can be easily palpated on the abdomen, cranial to the gland. This branch penetrates the gland into the middle half, draining the medial part of the perisinusal venous circle of the udder (2). The latter can be easily catheterized percutaneously through the subcutaneous abdominal vein. A manual clamp of the MV allows the catheter to be directed toward the middle branch of the cranial mammary vein, especially if a wire guide is used with the catheter to improve firmness. There would have been anastomoses between the lateral and the middle branch of the cranial mammary vein, which would be small. Further trials should determine if the middle branch of the cranial mammary vein would be safe from any significant contamination with the EPV reversed flow when old cows are used.

CONCLUSIONS

Our results suggest that there is a reversed blood flow from the EPV into the MV of the two third- and one fourth-lactation cows used in this study. Blood flow in the EPV appears to occur in the opposite direction in the four cows in second lactation used in this study. The first experiment demonstrated that the venous pathway taken by a pulse dose of PAH injected into the EPV was away from the udder in cows in second lactation, whereas the pathway was reversed in a third lactation cow. A second experiment showed that there was a backflux of the EPV toward the MV in two cows in third or fourth lactation but not in two cows in second lactation. Even though a small number of animals were used, the two different approaches used in the present study with different animals yielded the same conclusion. This supports Linzell’s hypothesis that some physiological modifications of mammary venous circulation occur when the mammary arterial blood flow increases along with milk production (15). Consequently, nonfunctional valves in the EPV have a direct effect on the representativeness of venous samples taken from the MV. In studies measuring the metabolic utilization of nutrients across the mammary tissue, cows in first and second lactations should preferably be used. The valvular incompetency in the EPV would likely bias the determination of MV nutrient concentration in older dairy cows, when the MV blood will be sampled into the lateral branch of the cranial mammary vein. This will lead to an increased variability and misinterpretation of mammary gland metabolism.

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