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Short Communication

Evaluation of perfused bovine udder for gene expression studies in dairy cows

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ABSTRACT. Intramammary infections are one of the main causes of productivity loss in dairy cows. To better understand the immune system response and to avoid the use of live animals, we validated the use of isolated bovine udder as an *ex situ* model. Six mammary glands were collected from cows ready for culling. Three udders were perfused with Tyrode's solution and three were not-perfused. During six hours, we collected perfusate samples for biochemical analysis. We also collected alveolar and teat canal tissue to evaluate gene expression. The biochemical parameters indicated that the perfused udders remained viable for the entire period of the experiment. A real-time polymerase chain reaction showed an increase in *18S rRNA* gene expression in the alveolar tissue at 3 and 4 h after perfusion. There was also an increase in the *Ubiquitin* gene in the teat canal from not-perfused udders at 1, 3, and 4 h after slaughter. In general, gene expression was stable

during the experiment. Our results indicated that the isolated perfused bovine udder model is appropriate for genetic studies, opening a new perspective in animal experimentation methods.

Key words: *Ex-situ* model; Genes; Mammary gland

INTRODUCTION

Owing to ethical issues, the use of *in vivo* models represents an important barrier to be transposed in animal studies. Therefore, *in vitro* models are widely used as an alternative (Kietzmann et al., 1993). In Brazil, the National Council for Animal Experimentation Control - CONCEA - recognized 17 alternative methods for animal experimentation studies (Brasil, 2014). The validation of new methods is of great interest. One of these new methods, the isolated perfused bovine udder approach, has been used in studies of mammary gland metabolism (Massart-Leën et al., 1986; Ehinger et al., 2006) and cutaneous absorption of drugs and cosmetics (Bäumer and Kietzmann, 2001). Despite of its potential, the method has not been widely used to evaluate the response against pathogens. This approach would be highly relevant, especially considering gene expression, where various parameters can be measured over time without using live animals models. In particular, the use of the isolated perfused bovine udder approach would be advantageous to investigate the response of the immune system during the first hours after challenge by pathogens, which is essential to complement *in vitro* studies. The reduction of cost, the non-use of live animals, as well as the reduction of milk production loss and even animal sacrifice, represent other advantages of this method. Another advantage is the use of mammary glands collected from slaughterhouses that are discarded after hitting and have no commercial value. However, in this case, a rigorous control is needed regarding sample collection and the general conditions of the animals.

In this study, we evaluated an isolated perfused bovine udder method for gene expression studies by using dairy cows scheduled for culling. To this end, five constitutive genes were evaluated and biochemical parameters of the perfused udder were annotated.

MATERIAL AND METHODS

The experiment was approved by the Animal Ethics Committee of the Embrapa Dairy Cattle (license no. 11/2011). Six dairy cows without history of udder diseases were selected from the herd of Campo Experimental José Henrique Bruschi, located in Coronel Pacheco, Minas Gerais State, Brazil. These animals, with healthy mammary glands, were selected among others scheduled for culling. Before slaughter, the animals were submitted to a veterinary examination (performed by three professional veterinaries), for the clinical evaluation of the udder (palpation) and the strip cup test. At seven and three days before slaughter, as well as on the day of slaughter, 72 milk samples were collected in 50 mL sterile tubes. They were refrigerated and sent to the Milk Microbiology Laboratory of Embrapa Dairy Cattle, located in Juiz de Fora, Minas Gerais, Brazil, for microbiological tests.

The udders were refrigerated at 8°C immediately after collection and transported in isothermal boxes to the Laboratory of Nanotechnology for Animal Health and Production of Embrapa Dairy Cattle. There, they were fixed, in the natural position, to a metal frame, using the proximally inserting skin and suspensory ligament. The time between collection and

perfusion of the udder in the laboratory was 40 min. The experiment was designed as described by Kietzmann et al. (1993) and Ehinger and Kietzmann (2000), using a perfusion fluid temperature of 37°C. For the viability tests, three udders were perfused with Tyrode's solution (NaCl 137 mM; KCl 2.7 mM; $CaCl_2.2H_2O$ 1.36 mM; $MgCl_2.6H_2O$ 0.50 mM; $NaH_2PO_4.H_2O$ 0.36 mM; $NaHCO_3$ 11.90 mM; Glucose 5.50 mM). The remaining three udders were placed without supply of oxygen or nutrients (not-perfused) during the experimental period.

Tissue samples were taken from the six udders (120 alveolar tissue samples and 120 teat canal samples). For the gene expression analyses, samples were collected from the four quarters of each udder at 0, 1, 3, 4, and 6 h after slaughter. The lactate dehydrogenase (LDH) activity and lactate production were also measured in the perfusate samples at 0 and 6 h after perfusion, to provide an indication of the conservation and oxygenation of the tissues (Kietzmann et al., 1993). These samples were stored at -20°C and sent to a commercial laboratory for analysis.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer instructions. The total RNA isolated was quantified by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was assessed by the RNA Integrity Number (RIN) after analysis on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) and eight samples were discarded due to RNA degradation (RIN values below 5.0). First cDNA strand was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA, USA) and the cDNA was stored at -20ºC until the real-time polymerase chain reaction (PCR).

Since gene expression can be affected by external stimuli, such as an absence of oxygen and nutrients (Siqueira, 2009), we choose the main endogenous control genes described in the literature to validate the stability of the udder when subjected to perfusion. Among those, there are genes related to synthesis, degradation, and transport of proteins, such as *Ubiquitin*, *RPLP0* (ribosomal protein, large, P0), *HPRT* (hypoxanthine-guanine phosphoribosyltransferase) and *18S rRNA* (Hochstrasser, 2009), in addition to others related to the transport of electrons and the respiratory chain, such as the *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) gene (Nicholls et al., 2012).

Expression was compared to *Ubiquitin*, *18S rRNA*, *HPRT*, *GAPDH* and *RPLP0* genes in the tissue samples collected from the six bovine udders totaling 232 samples. The real-time PCR was carried out using the SYBR Green® PCR Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer recommendations, optimizing the conditions for each primer (Table 1). After 40 amplification cycles, the dissociation curve was plotted to assure that each reaction produced a single fragment.

Table 1. Concentrations used of cDNA and primer for each gene and tissues evaluated.

Tissue	Gene	Primer (nM)	cDNA (ng)	Reference
Alveolar tissue	*18S rRNA*	600	100	Wang et al., 2005
	GAPDH	100	100	Mount et al., 2009
	HPRT	300	100	Tao et al., 2004
	RPLP0	200	100	Mount et al., 2009
	Ubiquitin	150	100	Singh et al., 2008
Teat canal	*18S rRNA*	600	200	Wang et al., 2005
	GAPDH	100	200	Mount et al., 2009
	HPRT	50	200	Tao et al., 2004
	RPLP0	200	200	Mount et al., 2009
	Ubiquitin	200	200	Singh et al., 2008

The data from the real-time PCR runs, generated by the ABI Prism 7300 Sequence Detection Systems, were exported to Excel files for analysis. The coefficient of variation between duplicates was calculated and those with values above 5% were repeated.

The efficiency of the reactions was calculated using REST© 2009, developed by Pfaffl et al. (2002) and Qiagen (available at http://www.gene-quantification.de/rest-2009.html). The expression stability of the five genes was evaluated using geNorm software (Vandesompele et al., 2002). Two out of five genes with the lowest expression variability and the greatest stability (*RPLP0* = 0.064 and *HPRT* = 0.067) for each analyzed tissue were selected as endogenous references to normalize the results.

Glucose and lactate concentration and LDH activity from the perfusate samples were analyzed with paired t-tests using SAS (SAS Institute Inc., 2008). The real-time PCR data were analyzed using REST© 2009, to compare the gene expression levels.

None of the animals had clinical mastitis and no clots were observed in milk samples analyzed by the strip cup test. However, 15 milk samples were contaminated with *Corynebacterium* sp. (data not shown). Although these microorganisms can affect the viability of the glands, the samples were not discarded in advance because this microbe is a secondary pathogen that is often present in the mammary glands of lactating cows (Gonçalves et al., 2014).

To check the viability of the three perfused udders, we measured the concentrations of glucose and lactate, and LDH activity. The samples were collected at the start (0 h) and end (6 h) of the experiment and the udders were considered viable if these three parameters remained relatively constant. The difference in the concentrations of glucose (0 h: 243 ± 37.65; 6 h: 250 ± 57.53) and lactate (0 h: 38.7 ± 13.59; 6 h: 30.56 ± 18.83), and LDH activity (0 h: 18.33 ± 23.15; 6 h: 41.67 ± 29.17) were not significant ($P < 0.05$) suggesting that the perfused udders were in stable conditions. These data also demonstrated that the contamination by *Corynebacterium* sp. did not affect the viability of the perfused udders during the evaluated interval.

The *HPRT* and *RPLP0* genes were selected as endogenous references to normalize the data once they showed the lowest expression variability both in the alveolar and teat canal tissues. The expression levels of *Ubiquitin*, *GAPDH*, and *18S rRNA* were analyzed in two kinds of tissue from the perfused (P) and not-perfused (NP) glands, comparing the observed values at 1, 3, 4, and 6 h after perfusion.

RESULTS AND DISCUSSION

In the alveolar tissue samples, we noted that, compared to at 0 h, the expression of *18S rRNA* showed a near three-fold increase at 4 and 6 h ($P < 0.05$, Figure 1A) in the perfused udders. This increase in gene expression might indicate that cell activity remained constant during the perfusion period, enabling continued RNA transcription and protein synthesis (Hayashi et al., 2012). This result can be explained by an increase in the number of ribosomes. It is also possible that nutrients remaining in the milk ducts and inside the glands could have maintained a stable cell activity that did not significantly affect gene function during the experimental period. Another reason that could explain the high viability of this tissue is the direct arrival of the Tyrode solution through the main artery used for perfusion. This may have more efficiently maintained the osmolarity and temperature, and hence the local cell activity. Therefore, this result is an indication that the alveolar tissue can remain stable for at least 6 h, the maximum time evaluated during this experiment. There was an increase in expression of *GAPDH* and *Ubiquitin* in the perfused udder samples during the experiment. However, compared to not-perfused udder samples, the difference was not statistically significant (Figures 1B and 1C).

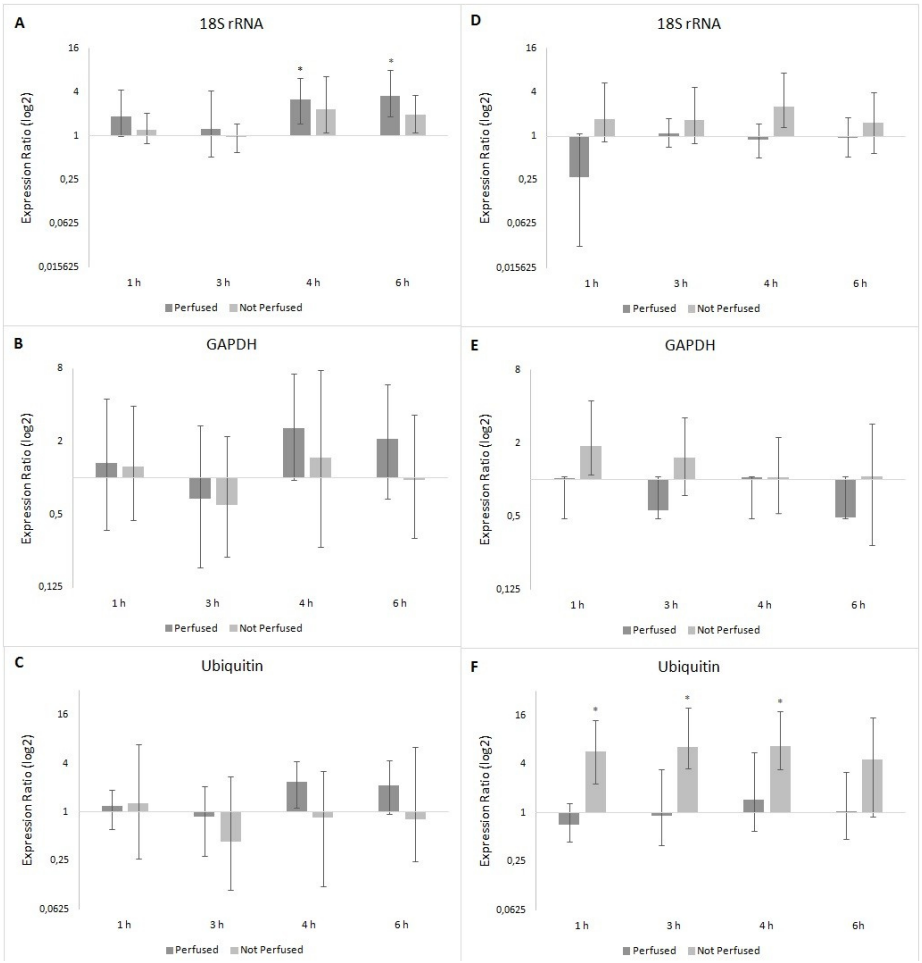

Figure 1. Expression levels of *18S rRNA*, *GAPDH*, and *Ubiquitin* genes in perfused and not-perfused udders evaluated at 1, 3, 4, and 6 h, using 0 h as a reference. **A-C**: alveolar tissue, **D-F**: teat canal tissue (*P < 0.05).

In the teat canal samples, the expression of *Ubiquitin* increased at 1, 3, and 4 h in the not-perfused udders (Figure 1F), while the variation in expression of *18S rRNA* and *GAPDH* were not significantly different (Figures 1D and 1E). The alterations in *Ubiquitin* expression might indicate a cell cycle deregulation associated with the necroptosis process (Iwai et al., 2014). The teat canal needs its basic physiological functions, such as peripheral circulation, to be active and maintain osmolarity. Therefore, the arrival of nutrients and tissue oxygenation may have contributed to this tissue being more sensitive to gene expression.

In general, in the not-perfused udders, the absence of oxygen seems to have changed the basal functions of the analyzed genes causing expression-level changes. In the perfused udders, these functions were maintained, indicating the viability of the perfusion model to keep the glands in physiological good condition. We also observed that the perfusion response was different for each tissue and therefore, it is important to evaluate each one separately to validate gene expression.

Our results suggest that the isolated perfused bovine udder model is appropriate for gene expression studies in alveolar tissue, since, in general, the evaluated samples showed stability for the constitutive genes. Thus, it can be concluded that the isolated perfused bovine udder may be a suitable model for genetic studies of the initial response to chemical and infectious agents, avoiding the use and possible impairment of the welfare of producing animals.

Conflicts of interest

The authors declare no conflict of interest.

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