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RESEARCH ARTICLE

Cystic ovary disease impairs transport speed, smooth muscle contraction, and epithelial ion transport in the bovine oviduct

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Abstract

Cystic ovary disease (COD) is a common cause of bovine infertility but the impact of this disease on the oviduct is unknown. The aim of this study was to analyze the effects of COD on particle transport speed (PTS), ciliary beat frequency, myosalpinx contraction, and epithelial ion transport. Oviducts were obtained from cows affected by COD and compared with those of healthy, mid-diestrus cows. PTS and CBF were examined using live-cell imaging. Smooth muscle contraction and epithelial ion transport were investigated using organ baths and Ussing chambers. Our results showed that muscarinic receptors are involved in cholinergic signaling in the oviduct and that forskolin-induced cyclic AMP production is involved in active ion transport in the oviductal epithelium. Oviducts from cows with luteal cysts revealed significantly decreased PTS ($p = 0.02$). Further to that, in the oviducts of COD cows, the cholinergic regulation of smooth muscle contractions and active epithelial ion transport were significantly diminished ($p < 0.0001$). These results imply that in COD cows, oviductal transport is compromised by decreased fluid flow speed and reduced cholinergic regulation of smooth muscle contraction and ion transport. This knowledge contributes to a more comprehensive understanding of COD supporting the development of novel therapeutic concepts for infertility treatment.

KEYWORDS
bovine oviduct, cholinergic regulation, cystic ovary disease, particle transport speed, smooth muscle contraction

1 | INTRODUCTION

Cystic ovarian disease (COD) is one of the most common reproductive diseases in dairy cattle (Yimer et al., 2018). Incidences in slaughtered cows range from 4.6% (UK, Millward et al., 2019) to 14.25% (Algeria, Mimoune et al., 2016). Ovarian cysts are defined as nonovulatory follicular structures, which measure 25 mm or more and persist for at least 10 days in the absence of a functional corpus luteum (CL) (Lüttgenau et al., 2016). Ovarian cysts may manifest as thin walled (≤ 3 mm) follicular cysts (FC) or thick-walled (> 3 mm) luteal cysts (LC) (Noakes et al., 2001; Vanholder et al., 2006). Plasma progesterone (P4) concentrations greater than 1.0 ng/ml point to the presence of an LC, whereas a P4 concentration less than 1.0 ng/ml denote an FC (Noakes et al., 2001; Vanholder et al., 2006). A recent

publication reported serum P4 concentrations to be 8–9 ng/ml in cows with LC and 0–1 ng/ml in cows with FC, which were similar to levels reported for healthy diestrus (10–11 ng/ml) and estrus (0–1 ng/ml) cows, respectively (Brodzki et al., 2019). Serum estradiol concentrations were reported to be 0–5 pg/ml in cows with LC and 15–20 pg/ml in cows with FC (Brodzki et al., 2019), however, concentrations as high as 50 pg/ml have also been reported (Mutlag et al., 2015).

The pathogenesis of COD is based on a neuroendocrine dysfunction of the hypothalamic–pituitary–gonadal axis (Noakes et al., 2001). Thus, an aberrant pre-ovulatory LH surge and altered steroid production are involved in cyst formation (Mimoune et al., 2017). Another underlying mechanism is the unresponsiveness of follicular cells to the LH surge and lower LH receptor numbers within granulosa and theca cells (Shimizu et al., 2018). Furthermore, cellular and molecular changes in growing follicles may play a role as an intrafollicular component of COD pathogenesis (Çolakoğlu et al., 2020; Stassi, Gareis, et al., 2019; Stassi, Gasser, et al., 2019). COD generates economic losses to the dairy industry due to extended calving intervals and treatment is required to help mitigate these losses (Smith, 2015). It is recommended that FC are treated with gonadotropin-releasing hormone (GnRH) analogs and that LC are treated with prostaglandin (PG) F2α (Bors et al., 2018). When cyst differentiation is not possible, GnRH is recommended over PGF2α (Bors et al., 2018).

The ovarian component of COD is well described in the literature, however, its impact on the rest of the genital tract is unknown. The oviduct plays a pivotal role in gamete transport, fertilization, and early embryonic development, and the timely transport of the embryo to the uterus for implantation is a prerequisite for successful pregnancy (Dixon et al., 2019). Oviductal smooth muscle contractions, ciliary beating, and the flow of the oviductal fluid provide the propulsive forces necessary for gamete and embryo transport (Croxatto, 2002). Together, these forces result in particle transport speed (PTS) which characterizes the transport of the oocyte and the embryo within the oviduct (Croxatto, 2002).

In cows, oviductal smooth muscle contractions are regulated by steroid hormones, neurotransmitters, and PGs (Kotwica et al., 2003; Wijayagunawardane et al., 2001). One of the best-known neurotransmitters, acetylcholine, is released from cholinergic nerves and has a direct contractile effect on the oviduct (Jankovic et al., 2004; Rajkumar & Sharma, 1981). To date, there are no data on the role of the cholinergic nervous system in oviductal motility in cows with COD, despite this system being dysregulated in several human pathological conditions of the reproductive system including tubal ectopic pregnancy and pre-eclampsia (Beckmann & Susanne, 2013). Hormonal and neuronal factors are also involved in the regulation of ciliary beating in the oviduct. Ciliary beat frequency (CBF) is increased by estradiol and decreased by progesterone (Nishimura et al., 2010; Wessel et al., 2004). CBF is decreased in oviducts from cows with severe salpingitis (Owhor et al., 2019) and in fallopian tubes from women with moderate and severe tubal endometriosis (Xia et al., 2018). However, to date, there are no data on the effect of COD on ciliary beating, which contributes to tubal fluid flow and PTS (Niwa et al., 2012; Noreikat et al., 2012). Previous research has shown that PTS in the oviduct is affected by lipopolysaccharide (O'Doherty et al., 2016) and adrenomedullin (Yoshimoto et al., 2017). Further to that, the mean coefficient of variation of PTS has been shown to be significantly increased in oviducts from cows affected by moderate and severe inflammation (Owhor et al., 2019). However, data on the effect of COD on PTS are still lacking.

Physiological oviductal fluid secretion from the secretory cells of the tubal epithelium is essential for creating an appropriate ionic environment ensuring healthy reproductive function (Keating et al., 2019; Leese et al., 2001). In cows, oviductal fluid secretion is driven by chloride secretion which is regulated by extracellular ATP and purinergic signaling (Keating & Quinlan, 2008; Keating et al., 2019). To date, data on the effect of COD on oviductal fluid secretion are still lacking, despite dysregulated fluid formation being a key feature of reproductive disorders such as hydrosalpinx (Ajonuma et al., 2005). Therefore, in this study, active ion transport across oviductal epithelium was measured in cows with and without COD using the Ussing chamber short-circuit current (SCC) technique.

Overall, the contractions of the tubal smooth muscle along with ciliary beating and tubal fluid flow occur in an optimal synchronized manner for successful fertilization and subsequent pregnancy (Dixon et al., 2019). To date, data elucidating the effects of COD on the oviduct, which exerts vital functions in gamete transport, fertilization, and early embryonic development, are lacking. It is important to address this paucity of literature to better understand the infertility issue that accompanies COD in cattle, and to further explore the causes for the low conception and pregnancy rates (Douthwaite & Dobson, 2000). The lack of literature in this area highlights the importance of clarifying the basic principles of tubal transport, including its regulation. We investigated many different functional features of the bovine oviduct aiming to establish a pilot study creating the basis for novel experimental approaches. Furthermore, therapeutic approaches to restore fertility in cows with COD have often been unsuccessful as even after successful induction of ovulation infertility has been reported to persist (Douthwaite & Dobson, 2000). This highlights the necessity of establishing more comprehensive therapeutic approaches which take the effects of COD on tubal ciliary beat frequency, PTS, smooth muscle contraction, and epithelial ion transport into account.

2 | RESULTS

2.1 | Effect of COD on tubal particle transport speed (PTS) and ciliary beat frequency (CBF)

PTS, which characterizes the speed of oocyte and embryo transport, was assessed using live-cell imaging. Polystyrene beads (Figure 1a, circles) were added to the buffer and were automatically tracked between adjacent mucosal folds (Figure 1a, arrows depict tubal folds, see Movie S1). Initially, it was investigated whether PTS was

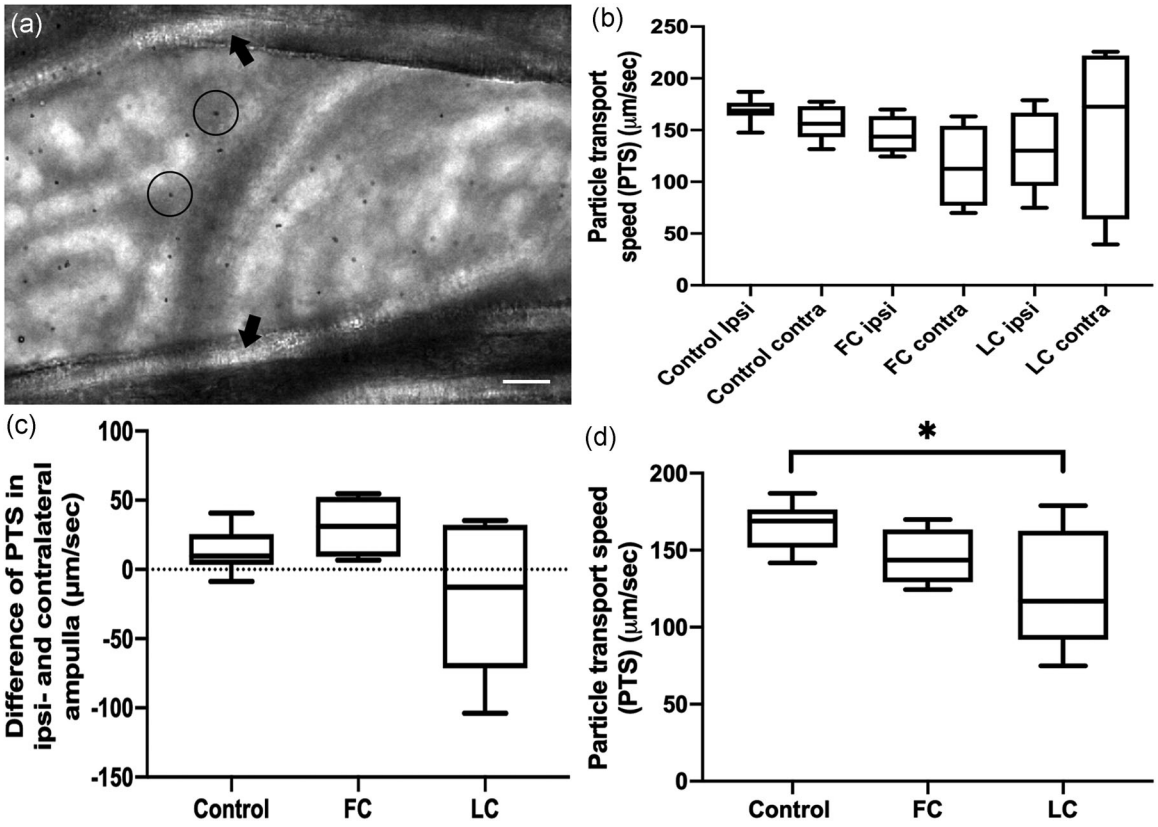

FIGURE 1 Effects of COD on tubal particle transport speed (PTS). (a) For assessment of PTS, polystyrene beads (circles) were tracked between adjacent mucosal folds (arrows, see Movie S1) (scale bar: 20 μm). (b) PTS was not significantly different in ipsi- and contralateral ampullae of control cows, cows with FC and cows with LC ($p = 0.07$, $p = 0.07$ FC, $p = 0.42$ LC, respectively, paired Student *t*-test). (c) When comparing the difference of PTS in ipsilateral and contralateral ampullae, the differences were not statistically different in controls and in cows with FC and LC (control vs. FC $p = 0.7$, control vs. LC, $p = 0.22$, ANOVA with Dunnett's multiple comparisons test). However, the coefficient of variation was significantly increased in cows with LC compared with the controls (control vs. LC $p = 0.001$, control vs. FC $p = 0.198$, FC vs. LC: $p = 0.016$, Levene's test for equality of variances). (d) PTS was significantly decreased in ipsilateral ampullae from cows with LC compared with ipsilateral ampullae from control cows ($p = 0.02$, ANOVA with Tukey's multiple comparisons test). Data are presented as box and whiskers Min–Max. Significance: $^*p < 0.05$. ANOVA, analysis of variance; COD, cystic ovarian disease; FC, follicular cysts; LC, luteal cysts

different between the ipsilateral and contralateral ampulla of each group. The ipsilateral ampulla is located at the same side as the ovary revealing a functional corpus luteum in control cows and a cyst in COD cows. There was no difference in PTS between ipsi- and contralateral ampullae of control (mid-diestrus) cows ($n = 14$, $p = 0.07$, paired Student *t*-test). Therefore, the side of ovulation did not influence PTS. Similarly, PTS was not significantly different between ipsi- and contralateral ampullae of cows with FC ($n = 8$) and cows with LC ($n = 12$) (FC $p = 0.07$, LC $p = 0.42$, paired Student *t*-test) (Figure 1b). However, when the difference in PTS between ipsi- and contralateral ampullae was plotted and the variance of PTS difference was compared, the coefficient of variation was significantly increased in cows with LC compared with the controls (control vs. FC $p = 0.198$, control vs. LC $p = 0.001$, and FC vs. LC: $p = 0.016$, Levene's test for equality of variances) (Figure 1c). Furthermore, when comparing ipsilateral ampullae only, PTS was significantly decreased in ampullae from cows with LC ($n = 7$, 124.5 ± 14.48 μm/s) compared with ampullae from control cows ($n = 8$, 166.3 ± 5.34 μm/s) ($p = 0.02$, analysis of variance [ANOVA] with Tukey's multiple comparisons test) (Figure 1d). PTS was also reduced in ampullae from cows with FC, but the difference was not significantly different from controls ($n = 4$, $p = 0.43$, ANOVA with Tukey's multiple comparisons test) (Figure 1d).

PTS is a result of ciliary beat frequency, smooth muscle contraction, and tubal fluid flow. Therefore, as a next step, CBF was assessed using live-cell imaging by quantifying the differences in the shades of gray produced by the beating cilia (Figure 2a, see Movie S2). When comparing ipsilateral and contralateral ampullae, CBF was similar in control cows ($n = 8$) and cows with FC ($n = 6$) and LC ($n = 14$) (control $p = 0.74$, FC $p = 0.49$, LC $p = 0.63$, paired Student *t*-test) (Figure 2b).

When comparing ipsilateral ampullae only, CBF values were not significantly altered in ampullae from cows with FC ($n = 4$, 16.9 ± 1.12 Hz) and LC ($n = 10$, 17.6 ± 0.92 Hz) as compared with ampullae from control, mid diestrus cows ($n = 5$, 15.6 ± 2.69 Hz) ($p = 0.83$ and $p = 0.55$, respectively, ANOVA with Dunnett's multiple comparisons test) (Figure 2c).

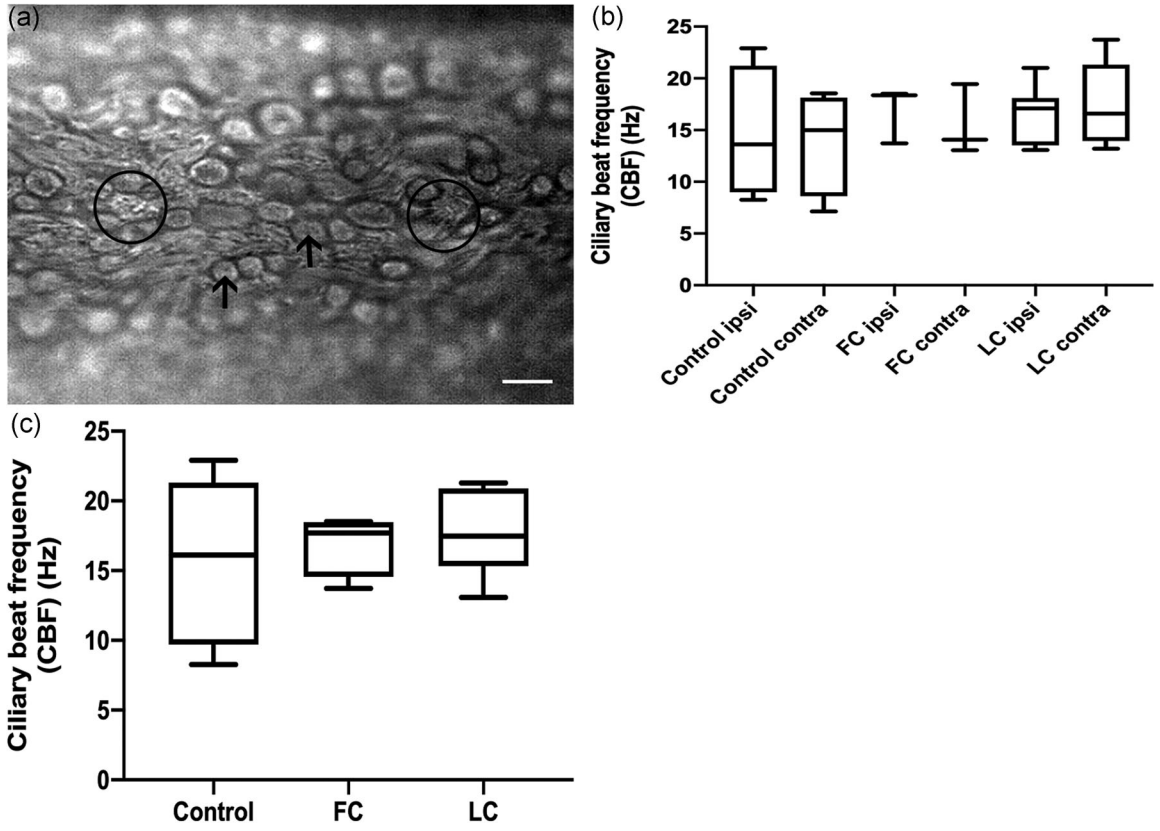

FIGURE 2 Effects of COD on oviductal ciliary beat frequency (CBF). (a) For CBF assessment, motile cilia (circles, see movie S2) of ciliated cells, interspersed among secretory cells (arrows, see movie S2), were analyzed (scale bar: 10 μm). (b) CBF was not significantly altered in ipsi- and contralateral ampullae from controls and cows with FC and LC (control $p = 0.74$, FC $p = 0.49$, LC $p = 0.63$ paired Student *t*-test). (c) CBF was similar in the ipsilateral ampullae from control cows and cows with FC and LC ($p = 0.83$ and $p = 0.55$, respectively, ANOVA with Dunnett's multiple comparisons test). Data are presented as box and whiskers Min–Max. ANOVA, analysis of variance; COD, cystic ovarian disease; FC, follicular cysts; LC, luteal cysts

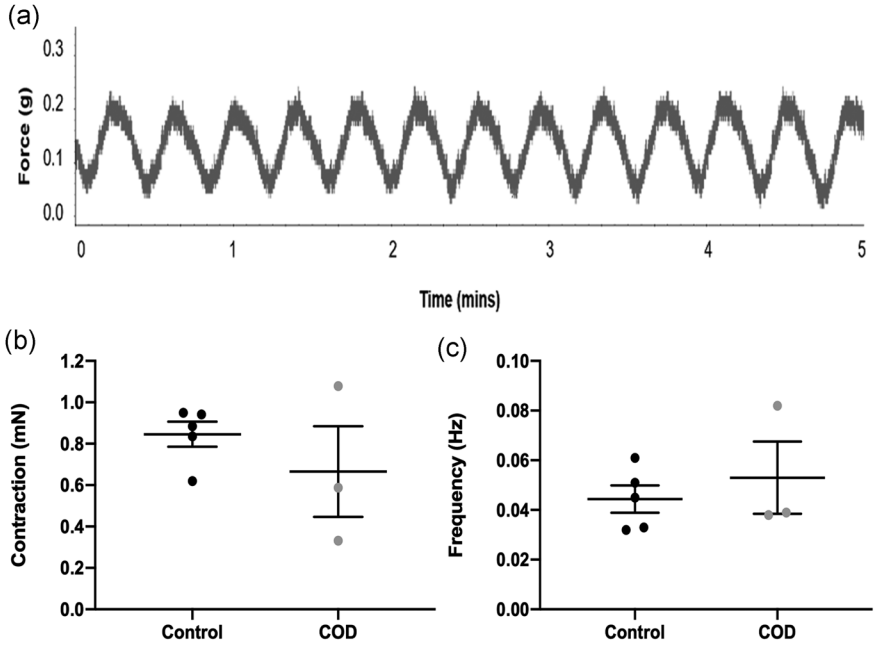

FIGURE 3 Effects of COD on amplitude and frequency of oviductal spontaneous smooth muscle contractions. (a) Spontaneous smooth muscle contractile activity in the healthy bovine oviduct reached up to 0.2 g. (b) The amplitude of spontaneous contractions was similar in ampullae from control cows and cows with COD ($p = 0.35$, unpaired Student *t*-test). (c) The frequency of spontaneous contractions was not significantly altered in ampullae from control cows and cows with COD ($p = 0.53$, unpaired Student *t*-test). Data are presented as the mean ± *SEM*. COD, cystic ovarian disease; *SEM*, standard error of mean

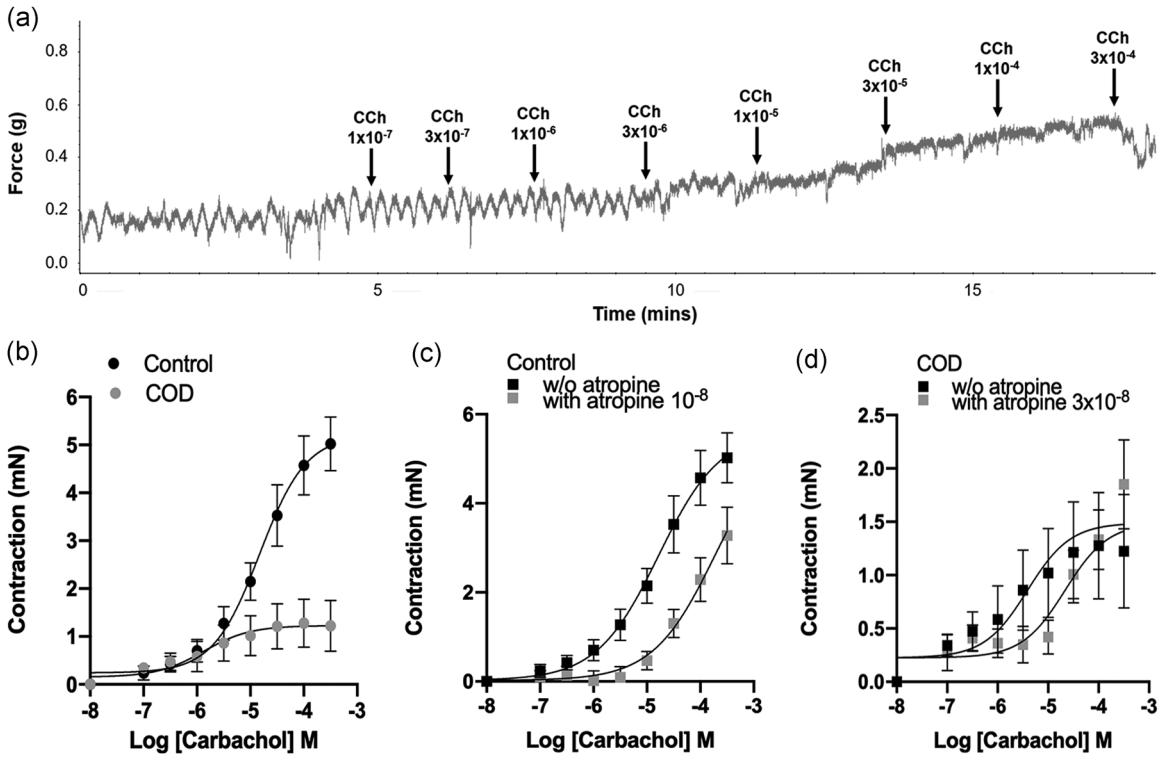

FIGURE 4 Effects of COD on cholinergic regulation of oviductal contractility. (a) Addition of carbachol (CCh, 1×10^{-7} M to 3×10^{-4} M) triggered ampullar smooth muscle contractile activity. Concentrations are indicated in M. (b) The concentration–response curve to CCh was significantly reduced in ampullae from cows with COD in comparison to that from control cows ($p = 0.0001$, nonlinear regression "omparison of fits" analysis). (c) Pretreatment with atropine shifted the CCh concentration–response curve to the right in ampullae from control cows ($p = 0.0001$, nonlinear regression "comparison of fits" analysis). (d) Pretreatment with atropine shifted the CCh concentration–response curve to the right in ampullae from COD cows ($p = 0.0001$, nonlinear regression "comparison of fits" analysis). Data are represented as the mean ± *SEM*. COD, cystic ovarian disease; *SEM*, standard error of mean

2.2 | Effect of COD on oviductal smooth muscle contraction and its cholinergic regulation

Organ baths were used to record smooth muscle contractility in the oviduct. In a first step, amplitude and frequency of spontaneous contractions were compared in control mid-diestrus cows ($n = 5$) and cows with COD ($n = 3$). Spontaneous contractions in the bovine ampulla occurred approximately every 20 s with a force reaching up to 0.2 g (Figure 3a). The average amplitude of spontaneous contractions in ampullae from control cows was 0.846 ± 0.06 mN (Figure 3b). COD did not significantly alter the amplitude (0.665 ± 0.22 mN) ($p = 0.35$, unpaired Student *t*-test) (Figure 3b). The average frequency of spontaneous contractions was similar in ampullae from control cows (0.04 Hz ± 0.01) and cows with COD (0.05 Hz ± 0.01) ($p = 0.53$, unpaired Student *t*-test) (Figure 3c).

In a second step, the aim was to clarify whether the cholinergic nervous system is involved in the regulation of bovine ampullar contractility. For this purpose, carbachol (CCh), which is a cholinomimetic drug, was added to the organ bath buffers. Addition of CCh in increasing concentrations (10^{-7}–10^{-4} M) to the ampullar tissue resulted in a gradual increase in amplitude in control and COD cows (Figure 4a, control). The response to CCh was significantly reduced in ampullae from COD cows ($n = 3$) as compared with control cows ($n = 6$) ($p = 0.0001$, nonlinear regression "comparison of fits" analysis) (Figure 4b).

In a third step, it was investigated whether CCh was acting via muscarinic cholinergic receptors. For this purpose, atropine, which is a muscarinic receptor antagonist, was added to the organ bath buffers. Atropine concentrations of 1×10^{-8} M (Figure 4c) and 3×10^{-8} M (Figure 4d) were applied, which was the range of atropine dosage with high efficiency. In the presence of atropine, the cumulative response to CCh revealed antagonistic action in ampullae from control, diestrus cows ($n = 5$) (Figure 4c) and COD cows ($n = 3$) (Figure 4d). Application of atropine resulted in a significant right-shift of the concentration–response curve to CCh in ampullae from control and COD cows ($p = 0.0001$, nonlinear regression "comparison of fits" analysis) (Figure 4c,d). Atropine caused a significantly reduced right shift in the dose–response in COD cows compared to controls ($p = 0.003$, generalized linear mixed model: group (control or COD): fixed effect, individual cow: random effect).

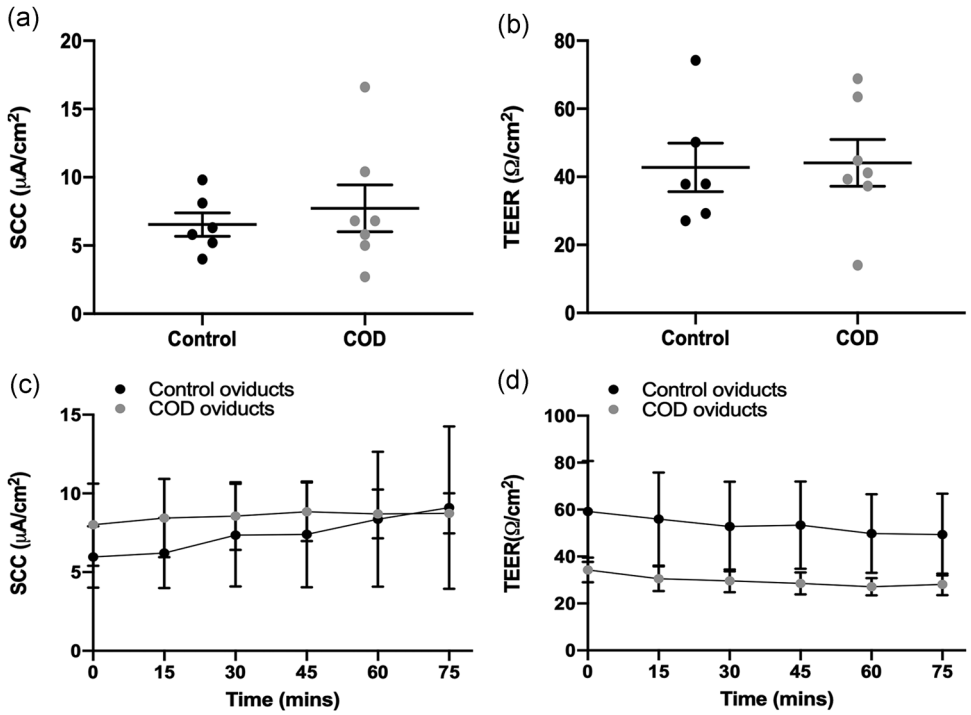

FIGURE 5 Effects of COD on bovine oviductal electrophysiology. (a) Baseline values for short circuit current (SCC) were similar in the epithelium of control cows and cows with COD ($p = 0.57$, unpaired Student *t*-test). (b) Baseline values for transepithelial electrical resistance (TEER) were similar in the epithelium of control cows and cows with COD ($p = 0.89$, unpaired Student *t*-test). (c) SCC and (d) TEER remained consistent over 75 min in control cows and cows with COD ($p = 0.61$ and $p = 0.88$, respectively, two-way ANOVA with Bonferroni correction). Data are represented as the mean ± *SEM*. COD, cystic ovarian disease; *SEM*, standard error of mean

2.3 | Effect of COD on tubal epithelial ion transport and its cholinergic regulation

In the first step, Ussing chambers were used to determine the baseline values of the short circuit current (SCC, measured in $\mu A/cm^2$) in the bovine oviductal epithelium. When the tubal mucosa was clamped to voltages varying from 1.5 mV to 2 mV below the spontaneous potential difference (PD), the change in PD was proportional to the applied current. In this way it was proven that the tubal epithelium acted as a linear resistor confirming the validity of the data obtained in the following experiments.

In a second step, the effects of COD on the baseline values for oviductal SCC and transepithelial electrical resistance (TEER) were investigated in control, mid-diestrus cows ($n = 6$) and cows with COD ($n = 7$) (Figure 5). The mean values for SCC in controls (6.5 ± 0.85 μA) and COD cows (7.7 ± 1.7 μA) were not significantly different ($p = 0.57$, unpaired Student *t*-test) (Figure 5a). Similarly, TEER in the tubal epithelium was not significantly altered by COD (controls 42.75 ± 7.12 Ω, COD 44.12 ± 6.85 Ω, $p = 0.89$, unpaired Student *t*-test) (Figure 5b). Basal SCC (Figure 5c) and TEER (Figure 5d) remained consistent over 75 min in the mucosa from COD cows ($n = 5$) and control cows ($n = 3$) ($p = 0.61$ and $p = 0.88$, respectively, two-way ANOVA with Bonferroni correction) (Figure 5c,d).

In a third step, CCh, which causes epithelial chloride secretion, was added to the buffer in the Ussing chambers. Using this drug, the effect of cholinomimetics on epithelial ion transport in the bovine ampulla of control ($n = 3$) and COD ($n = 3$) cows was investigated. Basolateral application of CCh (10^{-7}–10^{-4} M) induced an immediate transient, spiked and concentration-dependent increase in SCC followed by a return to baseline values (Figure 6a). The response (seen as change of SSC) to CCh was significantly reduced in the epithelium of COD cows compared to control cows ($p = 0.02$, unpaired Student *t*-test of area under the curve [AUC]) (Figure 6b).

In a fourth step, forskolin (FSK) was added to the buffer of the Ussing chambers. FSK activates the enzyme adenylyl cyclase which raises intracellular levels of cyclic AMP. Thus, this drug was used to investigate the effect of cyclic AMP on epithelial ion transport in the bovine ampulla of control ($n = 4$) and COD ($n = 3$) cows. Basolateral application of FSK (100 μM) induced a gradual and sustained response (Figure 6c). The change in SCC response to FSK was not significantly different in the epithelium of control and COD cows ($p = 0.23$, unpaired Student *t*-test) (Figure 6d).

3 | DISCUSSION

Using the bovine as a model, this study is the first to shed light on the effects of COD on the function of the oviduct. It is the first study to elucidate the effects of COD on transport speed, ciliary beating, and smooth muscle contraction in the tube. Each of these factors

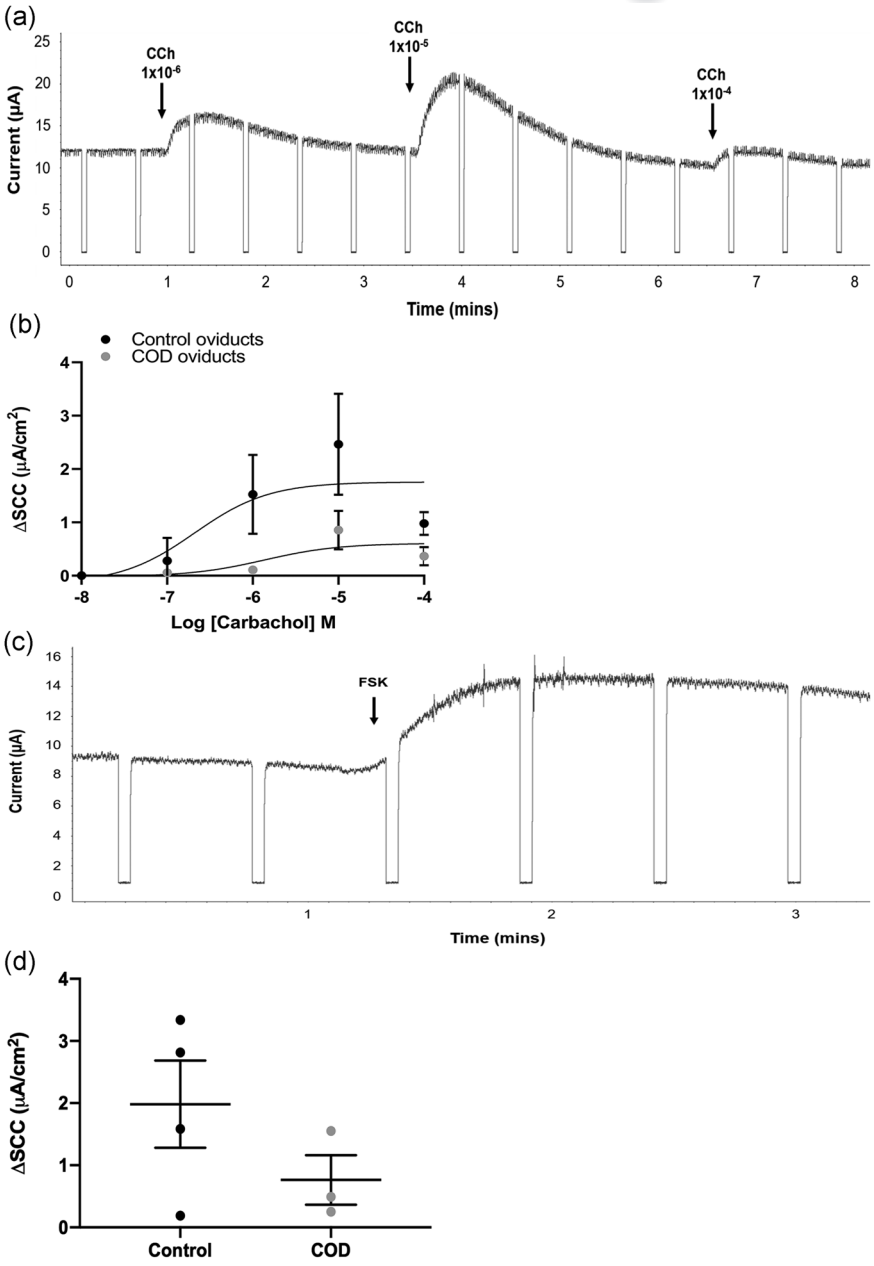

FIGURE 6 Effects of carbachol (CCh) and forskolin (FSK) on oviductal active ion transport. (a) Typical trace of oviductal active ion transport in response to carbachol (CCh). Concentrations are presented in M. (b) The concentration–response curve to CCh was significantly decreased in the epithelium of cows with COD in comparison to control cows ($p = 0.02$, unpaired Student *t*-test of area under the curve (AUC). (c) Typical trace of oviductal active ion transport in response to forskolin (FSK). (d) The change in short circuit current (SCC) in response to FSK was not significantly altered in COD cows ($p = 0.23$, unpaired Student *t*-test). Data are represented as the mean ± *SEM*. COD, cystic ovarian disease; *SEM*, standard error of mean

contributes to a precise and timely transport of the oocyte and early embryo—an essential prerequisite for the successful establishment of pregnancy (Kölle et al., 2009). Furthermore, the active epithelial ion transport in the healthy and COD oviduct is characterized. Active ion transport across the oviductal epithelium plays a major role in tubal fluid formation (Keating et al., 2019). Additionally, the ionic composition of oviductal fluid is crucial in providing a suitable environment for fertilization and early embryonic development (Chan et al., 2013). Most importantly, in this study, it is proven for the first time that bovine oviductal smooth muscle contractions and epithelial ion transport are regulated by the cholinergic system via muscarinic receptors and that a dysregulation of the cholinergic system occurs in COD.

For analysis of the transport of the oocyte and the early embryo in the healthy and COD oviduct, PTS was investigated. PTS is a result of ciliary beating (CBF), movement of tubal fluid, and smooth muscle contraction (Croxatto & Ortiz, 1975; Kölle et al., 2009). As shown by our quantitative live-cell imaging studies, PTS in the ipsilateral ampulla (located at the same side as the functional corpus luteum in the healthy cows and at the same side of the cyst in COD cows) and contralateral ampullae were not significantly different in healthy cows and cows affected by COD. These results point to the fact that systemic actions of hormones and other molecules are the more predominant modulators of PTS in healthy or diseased oviducts as compared to local effects. This is supported by the observation that in COD cows PTS in the ipsilateral oviduct was either higher or lower than in the contralateral oviduct. However, when comparing PTS in the ipsilateral ampullae of COD cows with control cows, it became obvious that cows with luteal cysts revealed significantly decreased PTS as compared with controls. It is possible that decreased

transport speed could reflect changes in hormone concentrations, specifically progesterone which is known to slow down oocyte transport (El-Banna & Sacher, 1976). However, previous reports of systemic serum progesterone levels are similar in diestrus cows (10–11 ng/ml) and cows with luteal cysts (8–9 ng/ml) (Brodzki et al., 2019). Future studies in this field should include hormone concentration analysis to gain a clearer understanding of the exact mechanisms surrounding dysregulated transport speed.

Further to that, our results revealed that in cows with luteal cysts the coefficient of variation of the difference of PTS in the ipsi- and contralateral ampulla was significantly higher in cows with luteal cysts compared to controls. Similarly, it has been shown in bovine inflammatory tubal disease that the coefficient of variation in PTS is significantly increased (Owhor et al., 2019) supporting the hypothesis that diseases of the female genital tract are associated with a high variance in the different factors modulating gamete and early embryo transport.

The observed decrease in PTS in cows with luteal cysts could be due to alterations in ciliary beat frequency, smooth muscle contraction, or fluid flow, which is influenced by active epithelial ion transport. Therefore, our further studies focused on elucidating these factors. As shown by our results, CBF was not the factor responsible for the decreased PTS observed in cows with luteal cysts. CBF was similar in ipsi- and contralateral ampullae and was similar in control and COD cows. In humans and guinea pigs, CBF has been documented to vary during the ovarian cycle (Critoph & Dennis, 1977; Lyons et al., 2002; Nishimura et al., 2010) and it has been shown that CBF is controlled by endogenously secreted beta-estradiol and progesterone (Nishimura et al., 2010). Further studies including serum hormone concentrations are required but unaltered CBF could reflect similar levels of progesterone and estrogen that have been reported in diestrus cows and cows with luteal cysts (Brodzki et al., 2019).

Recent studies have shown that smooth muscle contractions are likely to play a more significant role than ciliary beating for oviductal oocyte transport (Dixon et al., 2019; Raidt et al., 2015). When comparing the tubal smooth muscle contraction in controls and COD cows, the amplitude and frequency of the contractions were similar. These results are supported by studies in the bovine oviduct which report consistent values for frequency and amplitude of spontaneous contractions throughout follicular and luteal phases of the estrus (Isla et al., 1989; Kotwica et al., 2003; Troedsson et al., 1995). This has also been reported in humans during the menstrual cycle (Jankovic et al., 2004). Regarding healthy control cows, the data obtained in this study provide the first evidence that the cholinergic nervous system is involved in the regulation of bovine oviduct contractility. To date, a cholinergic regulation of tubal function has only been shown in the human (Jankovic et al., 2004) and in the rabbit (Rajkumar & Sharma, 1981). According to our study the supplementation of CCh, which mimics acetylcholine, causes an increase in the contractility of the healthy bovine oviduct in a concentration-dependent manner. The fact that atropine is able to block the effect of CCh indicates the presence of muscarinic receptors. Similarly, the presence of muscarinic receptors has previously been described in the murine oviduct (Wolff et al., 2012) and in the human Fallopian tube (Jankovic et al., 2004). In COD cows, the response to CCh is significantly diminished but atropine is still effective at delaying the response. Thus, oviducts from COD cows have the capacity to respond to cholinergic stimuli via muscarinic receptors—but it is reduced compared to control cows. Therefore, these results show that even though spontaneous contractions are unaltered in COD cows, the contractile activity of the oviduct is specifically impaired under cholinergic stimulation as compared to controls. Impaired smooth muscle contraction might be associated with dysregulated transport of the gametes and the early embryo.

Besides cholinergic control of smooth muscle contraction, the cholinergic regulation of active epithelial ion transport is also significantly diminished in cows with COD. Active ion transport across an epithelium plays a major role in fluid formation (Keating et al., 2019). In the bovine, fluid formation is driven by the active transport of chloride ions (Cl^-) which is reported to be maintained primarily through calcium activated Cl^- channels and cyclic AMP-activated cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels (Keating & Quinlan, 2012). In Ussing chambers, active ion transport is reflected by changes in SCC. CCh mediates active chloride secretion by inducing intracellular accumulation of Ca^{2+} by activation of both muscarinic and nicotinic receptors (Bader & Diener, 2015). Muscarinic receptors M1, M3, and M5 increase cytosolic concentrations of Ca^{2+} by activating phospholipase C which stimulates protein kinase C (Beckmann & Susanne, 2013). An increase in the concentration of cytosolic Ca^{2+} induces chloride epithelial secretion (Mahmood et al., 2001). Our results showed that FSK-induced cyclic AMP production is involved in active ion transport in the bovine oviductal epithelium of cows with and without cysts. Regarding oviducts affected by COD, it became obvious that the response to CCh is significantly diminished in oviducts from cows with COD, which may have a consequence for active ion transport and fluid formation in COD. This effect may be attributed to CCh solely, as baseline SCC, before the administration of drugs, is similar in oviducts from cows with and without COD, but also points to the presence of complex processes and the effects of multiple factors. Fluid secretion by the oviductal epithelium is essential for the transport as well as the nutrition of the gametes and the early embryo. Furthermore, the ionic composition of the oviductal fluid is crucial in providing a suitable environment for key reproductive events and early embryonic development. Therefore, alterations in its composition or the rate of its formation might result in a microenvironment that is unable to support fertilization and early embryonic development and transport.

In summary, this study is the first to describe the negative impact of COD on the transport function of the oviduct in real-time and under near in vivo conditions using a unique live-cell imaging technology. Furthermore, the effect of cholinomimetics on bovine tubal contractility and epithelial ion transport was investigated in the bovine oviduct for the first time. Our results showed that the PTS, which characterizes the speed of gamete and early embryo transport, is diminished in oviducts of cows affected by COD. Additionally, the cholinergic regulation of

oviductal contractility and epithelial ion transport is significantly reduced in oviducts of COD cows. Our results imply that oviductal transport function is dysregulated in cows with COD, which might explain why therapeutics have been largely unsuccessful in establishing pregnancy in cows with COD. Thus, this knowledge creates the basis for the establishment of novel, more comprehensive therapeutic strategies identifying cholinergic signaling as a promising target for rescuing transport function in cows with ovarian cysts.

4 | MATERIALS AND METHODS

4.1 | Ethics

This study did not require ethical approval as bovine tissues were obtained from the abattoir. Full ethical exemption was granted by the Office of Research Ethics, University College Dublin (AREC-E-18-48-KOELLE).

4.2 | Tissue collection

Oviducts from cows (breed: Holstein-Friesian, age: 3–6 years) with COD ($n = 28$) were obtained from the abattoir (Kildare Chilling Company) immediately after slaughter and compared to specimens obtained from healthy cows ($n = 24$). Experiments were performed within 2 h of collection. All healthy control samples used for this study were in mid-diestrus. This cycle stage was chosen to elucidate the differences between the molecular actions of a functional corpus luteum in the healthy individual and the aberrant steroidogenic nature of ovarian cysts in cows with COD. The presence of diestrus was determined by (1) the presence of a corpus luteum occupying more than 50% of the ovarian surface area, (2) closed cervix, and (3) absence of mucus in the uterine horn and cervix. Samples were excluded if there were signs of infection, inflammation, or pregnancy. Genital tracts were determined to be cystic if structures with a diameter greater than 25 mm were observed on one or both ovaries in the absence of a corpus luteum. FC had a thin wall (0.06 ± 0.01 cm) and did not reveal any signs of luteinization (yellow to orange colored tissue) when opened. In contrast, LC had a thick wall (0.28 ± 0.03 cm) and at least 50% of the internal cyst wall was luteinized revealing an intense yellow/orange color. In control and cystic genital tracts, the ipsilateral and contralateral oviducts were removed from the surrounding mesosalpinx. For Ussing chamber experiments, 1 cm portions of the infundibulum and first third of the ampulla were used, while 1 cm portions of the middle-third of the ampulla were used for live-cell imaging and organ bath experiments. The ampulla was used for all experiments as it is the site of fertilization and the first embryo-maternal communication, and therefore is pivotal for fertility and for the establishment of a successful pregnancy (Kölle et al., 2009). For analyses of epithelial ion transport, the infundibulum was also included due to its role in guiding the cumulus–oocyte complex into the oviduct after ovulation.

4.3 | Sample preparation for live-cell imaging

Quantitative analyses of PTS and CBF in bovine ampullae from mid-diestrus and cystic cows were performed using a digital live-cell imaging technology established in our lab (Kölle et al., 2009). Samples of ampulla (1 cm) were opened longitudinally and pinned onto Delta T dishes (Bioptech Inc.). Dishes were pre-coated with a 2 mm layer of silicone (Sylgard® 184, Dow Corning) and the ampullar tissue was fixed in place using 26 G Sterican® needles. All samples were washed with phosphate-buffered saline followed by 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (10 mM HEPES, 136.4 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2.2H_2O$, 1 mM $MgCl_2.6H_2O$, and 11 mM glucose). Specimens were incubated with HEPES buffer in a humidified chamber for 10 min. Once mounted, samples were maintained at 37.5 ± 1°C using a stage heater (Delta T Controller, Bioptech Inc) and an objective heater (Delta T Controller, Bioptech Inc).

4.4 | Quantitative analysis of PTS in the oviducts of COD and control cows

Initially, PTS was assessed within groups, whereby paired data from the ipsilateral and contralateral ampullae were compared in control, mid-diestrus cows ($n = 14$) and cows with FC ($n = 8$) and LC ($n = 12$). Second, PTS was assessed between groups, whereby unpaired data from ipsilateral ampullae only were compared between control cows ($n = 8$) and cows with FC ($n = 4$) and LC ($n = 7$).

One cm specimens were fixed in Delta T dishes, submerged in 1.8 ml HEPES buffer and mounted on a heated stage. Specimens were imaged with a ×20 water immersion objective (UMPLFL x20 W/0.5, Olympus). The videos were recorded using software from StreamPix® 7.0 (NorPix) linked to a SUMIX Mx7 camera (12 frames/s [FPS] mounted on a BX51W1 fixed-stage upright microscope [Olympus]). For PTS analysis, 3 µl of 2.8 µm diameter polystyrene beads (concentration 3×10^6/µl, Dynabeads, Life Technologies) were added to the buffer media. Videos were recorded documenting the flow of dynabeads between two oviductal folds which were in focus in three random areas of interest (AOI). For PTS analysis, each video was converted from 21-bit to 8-bit greyscale and a threshold was set to include only actively moving polystyrene beads. The beads were tracked automatically using ImagePro® software (MediaCybernetics) and exported to a tracking data excel table to calculate the mean transport speed.

4.5 | Quantitative analysis of CBF in the oviducts of COD and control cows

Initially, CBF was assessed within groups, whereby paired data from the ipsilateral and contralateral ampullae were compared in control, mid-diestrus cows ($n = 8$) and cows with FC ($n = 6$) and LC ($n = 14$). Second, CBF was assessed between groups, whereby unpaired data

from ipsilateral ampullae only were compared between control cows ($n = 5$) and cows with FC ($n = 4$) and LC ($n = 10$).

Specimens (1 cm) were fixed in Delta T dishes, submerged in 1.8 ml Hepes buffer and mounted on the heated stage. CBF was recorded in five randomly selected AOIs with motile cilia. Specimens were imaged with a ×40 water immersion objective (UMPLFL x40 W/0.8, Olympus). The videos were recorded using software from StreamPix® 7.0 (NorPix) linked to a SUMIX Mx7 camera (100 FPS) mounted on a BX51W1 fixed-stage upright microscope (Olympus). For CBF analysis, each video was converted to binary using ImageJ® (National Institutes of Health, USA) and a random region of beating cilia were selected using the rectangular selection tool. The differences in grayscale were converted to a frequency (Hz) using the Fast Fourier Transformation in AutoSignal® (Systat Software). Five random regions of beating cilia were analyzed per video and five videos were recorded per oviduct.

4.6 | Analysis of oviductal smooth muscle contraction in COD and control cows

Oviductal smooth muscle contraction was analyzed using organ baths. Isometric tension recordings were carried out on ampullae from mid-diestrus cows ($n = 5$) and cows with COD ($n = 3$; 2 LC, 1 FC). The discrimination between cyst type was abandoned in these experiments and a pilot study was carried out to elucidate the basic mechanisms of smooth muscle contractility in the oviduct. Rings of the ampulla, approximately 0.3 × 0.5 mm (length × width), were dissected and mounted in an 8-chamber organ bath system (10–15 ml volume per chamber, water-jacketed) (Myobath, World Precision Instruments, Inc). Organ baths containing Krebs–Henseleit physiologic salt solution (118 mM NaCl, 11.1 mM D-glucose, 24.9 mM $NaHCO_3$, 1.2 mM $MgSO_4.7H_2O$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2.2H_2O$, pH 7.4), were aerated with a gas mixture of 95% O_2 and 5% CO_2 and were maintained at 37°C. Once mounted, a defined amount of force (0.5–1 g) was applied to each piece of ampulla until the baseline tension reached 10 mN. This baseline tension determined the optimal length at which smooth muscle cells respond. Only specimens displaying spontaneous contractility for at least 60 min were used in the experiments. Specimens were equilibrated for approximately 60 min before being challenged with drug solutions. CCh, (a cholinomimetic agent) was used to assess the contractility of oviducts from cows with COD ($n = 3$) as compared with control oviducts ($n = 6$). A single dose was added (1×10^{-4} M) to test tissue viability. The baths were washed twice with aerated Krebs–Henseleit physiologic salt solution. This was followed by a 30 min stabilizing period. Atropine was added to the baths (1×10^{-8} M and 3×10^{-8}) to identify whether CCh was acting via muscarinic or nicotinic receptors. After 30 min, cumulatively increasing concentrations of CCh (10^{-7}–10^{-4} M) were added. All stock solutions of drugs were prepared according to the supplier's instructions and stored at −20°C. Drugs were diluted further immediately before each experiment from stock solutions using Krebs–Henseleit physiologic salt solution. The same protocol was carried out in each bath, except one bath in which atropine was not administered as a control. The contractile activity of the oviductal rings was recorded and analyzed using Power Lab software, Chart (version 5.0; AD Instruments Pty Ltd). For spontaneous activity, a 300 s window was selected before the addition of cumulatively increasing CCh. The amplitude of spontaneous contractions was assessed by measuring the peak-to-peak amplitude (height from trough to peak) during a further 300 s period. Amplitude was measured in grams force (g) and was converted to millinewtons (mN) using the 101.97 conversion factor. The frequency of spontaneous contractions was assessed by counting the number of peaks during 300 s period. Values for mean force were determined in g for each dose of increasing concentration of CCh and converted to mN using the 101.97 conversion factor. Data were displayed as mN because Newton is the International System of units for measuring Force.

4.7 | Analysis of ion transport in the oviductal epithelium of COD and control cows

Transepithelial ion transport was investigated using Ussing chambers. For Ussing chambers, a membrane is mounted between two halves of a chamber so that the mucosal membrane faces one side of the chamber while the serosal membrane faces the other. The principle of electrophysiological assessment of ion transport is to eliminate any chemical and electrical gradients across the epithelial preparation. This setup ensures that the current that flows can only be the net result of all the active transport processes of charged ions in one direction as a result of adding a substance (Clarke, 2009). The addition of drugs or hormones to the apical or basolateral chambers of mounted preparations result in a change in current, referred to as a change in SCC (Mahmood et al., 2001).

Oviducts from mid-diestrus cows ($n = 6$) and cows with COD ($n = 7$) were freed from surrounding mesosalpinx. Mucosal preparations of the infundibulum and ampulla were prepared by opening the oviduct longitudinally and pinning the tissue mucosa-side down on a dissection board. The serosa and smooth muscle layers were removed by blunt dissection using a size 5 watchmaker forceps. Stripping of the serosa and the muscular layer was performed to minimize the influence of the intrinsic neuromuscular system of the mounted sample (Clarke, 2009). Mucosal oviductal segments were then mounted in Ussing chambers with a circular window of 0.63 cm^2, bathed bilaterally with Krebs–Henseleit physiologic salt solution and continuously held in a gas mixture of 95% O_2 and 5% CO_2. The temperature of the chambers was maintained at 37°C by an outer glass water jacket connected to a heated recirculating water bath. Transepithelial potential difference (PD, mV) was recorded in an open circuit for a period of 20 min after which the tissue was short-circuited with the transepithelial potential difference clamped at zero using an epithelial voltage clamp (WPI, UK). Analog signals were digitized using a Powerlab data acquisition module and analyzed with Chart software (version 5.0; AD Instruments Pty Ltd).

For recording the short circuit current (SCC, μA/cm^2), the tissue was equilibrated for a further 30 min. The voltage clamp was intermittently opened for 3 s every 30 s and the change in current was used to calculate the resistance (Ω) using Ohm's law (V = IR), where V = voltage, I = current, and R = resistance. After a 30-min equilibration period, SCC was documented during the addition of increasing concentrations of CCh (10^{-7}–10^{-4} M) to preparations from mid-diestrus cows (n = 3) and cows with COD (n = 3). CCh, which stimulates chloride secretion, was added cumulatively to the basolateral chamber to investigate chloride-dependent ion transport in oviducts from cows with COD as compared with control oviducts. SCC responses to FSK (100 μM) were also recorded in preparations from mid-diestrus cows (n = 4) and cows with COD (n = 3). FSK, which activates adenylyl cyclase, was added to the basolateral chamber to investigate cyclic AMP-dependent ion transport in oviducts from cows with cysts as compared with control oviducts.

Values for baseline SCC were extracted directly from trace recordings at 4 AOIs during a 60 s interval before the application of drugs. Values for baseline TEER were calculated using Ohms law and expressed as Ω (ohms) × cm^2 by multiplying by the exposed tissue area (0.63 cm^2). Values for SCC were also extracted from trace recordings before and after the application of the drugs mentioned. Results were expressed as mean SCC change from basal (ΔSCC in μA)/0.63 cm^2 window area of chamber.

4.8 | Statistical analysis

All statistical analyses were performed using g SPSS 26.0 (IBM) and GraphPad Prism Software (version 8.0.0 for Windows). For all data, the normality of the distribution was checked using the Shapiro–Wilk test. Normally distributed data were presented as the mean values ± *SEM*. A paired Student *t*-test was used to compare PTS and CBF in ipsilateral and contralateral ampullae from control cows as well as cows with COD. For comparison of PTS in ipsilateral ampullae between groups, ANOVA with Tukey's multiple comparison test was used. For comparing the differences of PTS in ipsi- and contralateral ampullae of cows with follicular cysts, luteal cysts, and controls, ANOVA in combination with Dunnett's multiple comparison test was applied. Levene's test for equality of variances was used to compare the variances of the differences of PTS. The Unpaired Student *t*-test was used for the following comparisons: spontaneous smooth muscle contractions, transepithelial electrical resistance, and short circuit current in control cows and cows with COD; change in short circuit current in response to CCh and forskolin in control cows and cows with COD. Concentration–response curves to CCh in organ baths were compared in control cows and cows with COD using nonlinear regression "comparison of fits" analysis. For comparing the right shift in smooth muscle contraction in response to application of atropine in both control and COD cows, the generalized linear mixed model was used with the groups (control, COD) included as fixed effects and the individual cows included as random effects. The changes of the TEER over time were compared in control cows and cows with COD by using two-way ANOVA with Bonferroni correction. Differences were considered statistically significant if $p \leq 0.05^{*}$, $p \leq 0.01^{**}$, and $p \leq 0.001^{***}$.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ETHICS STATEMENT

This study did not require ethical approval as bovine tissues were obtained from the abattoir. Full ethical exemption was granted by the Office of Research Ethics, University College Dublin (AREC-E-18-48-KOELLE).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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