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In vivo measurement of pH and CO₂ levels in the uterus of sows through the estrous cycle and after insemination

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The pH–CO₂–HCO₃[−] system is a ubiquitous biological regulator with important functional implications for reproduction. Knowledge of the physiological values of its components is relevant for reproductive biology and the optimization of Assisted Reproductive Technologies (ARTs). However, in situ measurements of these parameters in the uterus are scarce or null. This study describes a non-invasive method for in situ time-lapse recording of pH and CO₂ within the uterus of non-anesthetized sows. Animals were at three different reproductive conditions, estrous with no insemination and two hours after insemination, and diestrous. From pH and CO₂ data, HCO₃[−] concentration was estimated. The non-invasive approach to the porcine uterus with novel optical probes allowed the obtaining of in situ physiological values of pH, CO₂, and HCO₃[−]. Variable oscillatory patterns of pH, CO₂ and HCO₃[−] were found independently of the estrous condition. Insemination did not immediately change the levels of uterine pH, CO₂ (%) and HCO₃[−] concentration, but all the values were affected by the estrous cycle decreasing significantly at diestrous condition. This study contributes to a better understanding of the in vivo regulation of the pH–CO₂–HCO₃[−] system in the uterus and may help to optimize the protocols of sperm treatment for in vitro fertilization.

Carbon dioxide (CO₂) and H₂O are the most common end products of the energy catabolic pathways in living organisms. In multicellular organisms, carbonic anhydrase mediates the reaction between these two molecules to deliver carbonic acid (H₂CO₃), which rapidly dissociates into a H⁺ and a bicarbonate ion (HCO₃[−]). The resulting equilibrium between CO₂, H⁺ and HCO₃[−] plays a pivotal role in the regulation of many relevant biological processes such as breathing, diuresis or reproduction^{1,2}.

The success of fertilization starts with sperm migration within an adequate microenvironment of the female genital tract. Both, H⁺ and HCO₃[−] have a pivotal role in regulating sperm fertilization capacity, which is progressively achieved along their trajectory from the epididymis to the fertilization site in the oviduct through a gradient of increased pH and HCO₃[−] concentrations^{3–5}. Both components are directly involved in many biochemical reactions resulting in the so called ‘sperm capacitation’, a process necessary for fertilization^{6,7}. HCO₃[−] and pH play also an important role for embryo transport, development and implantation^{2,8}. The production and secretion of HCO₃[−] and H⁺ in the uterus, although not fully elucidated, includes different exchangers^{9,10} which are sensitive to sex-steroids hormones^{8,10–13}.

Reference in vivo levels of pH, CO₂ and HCO₃[−] in the uterus and oviduct of several mammals (rabbit, monkey, human, bovine, equine or porcine) have been obtained during different stages of the estrous cycle (see Table 1), while other values were obtained from oviductal or uterine fluid samples^{14,15}. pH is the best characterized parameter. Uterus and oviduct pH vary between species (range from 6.83 to 7.35 and 6.7–8.3, respectively) and is influenced by the stage of the estrous cycle (Table 1). Contrastingly, no HCO₃[−] values have been recorded directly from the uterus, so the available data were obtained from oviductal fluid, either directly or from samples^{14–16}. Likewise, very few studies and with minimal explicit information have been aimed at determining CO₂ tension (pCO₂) within the uterus or oviduct^{14,16}. In the porcine species, pH values in the oviduct are very variable (ranged

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Species	Organ	Estrous cycle	pH	HCO ₃ ⁻ (mMol)	pCO ₂	References
Rabbit	Oviduct	–	7.94	–	–	24
Primate (<i>Macaca mulatta</i>)	Oviduct	Follicular phase	7.1–7.3	35*	–	16
		Luteal phase	7.5–8.0	90*	–	
		Menstrual cycle	–	–	89 Torr (46–143)	
Bovine	Uterus	Day 0 estrous cycle	7.22	–	–	23
		Day 1–6 estrous cycle	7.28–7.35	–	–	
	Uterus	Day 0 estrous cycle (estrous)	6.85–6.89	–	–	20,21
		Day 7 estrous cycle (diestrous)	7.03–7.15	–	–	
	Uterus	–	7.00–7.05	–	–	25
	Oviduct	Day 0–6 estrous cycle	7.41–7.60	–	–	19
	Uterus	Day 6–14 estrous cycle	6.96–7.21	–	–	
Equine	Uterus	Estrous	~6.83–6.97	–	–	22
Porcine	Oviduct	Estrous	7.02	–	–	17
	Uterus	Estrous	6.98	–	–	
	Oviduct	Pre-ovulatory phase	7.76–7.96	–	–	18
		Post-ovulatory phase	7.37–7.40	–	–	
	Oviduct	Estrous	6.7–8.3	–	–	15

Table 1. Summary of the studies measuring pH, CO₂ and/or HCO₃⁻ levels directly in the female genital tract (uterus and/or oviduct) of different species. *Calculated from CO₂ and pH values.

from 6.7 to 8.3) depending on the study and the stage of the cycle^{15,17,18}, while HCO₃⁻ levels of 10.0–33.1 mMol were estimated in samples of oviductal fluid retrieved under surgical conditions¹⁵. In the uterus, pH (6.98) was only measured in a preliminary study in a series of 6 gilts¹⁷, with no mention to CO₂ or HCO₃⁻ values.

The characterization of physicochemical parameters *in vivo* is always a challenge. The use of medication to suppress any pain or stress caused by the surgical approach or during the recording of data always has an impact on the physiological condition. Hugentobler et al.¹⁹ compared the influence of the anesthetic protocol on oviductal and uterine pH in cows. When intravenous anesthesia (thiopentone) was compared with inhalatory anesthesia (halothane) oviduct and blood pH was reduced, whereas uterus pH increased. For this reason, the most reliable results in this type of approaches are those with minimum or complete absence of pre-medication. This has been achieved in cows^{20,21} and mares²², where a non-invasive external approach to the uterine lumen via the cervix allowed direct pH measurements. However, until now a similar approach to the uterus has not been tried yet in pigs.

In most previous studies the *in vivo* measurement of uterine pH was carried out with potentiometric devices. For this, miniaturized pH glass electrodes either as a single unit or in combination with a reference electrode were extensively used^{15–25}. Similarly, for pCO₂ measurements a pH electrode was converted to a Severinghaus type electrode covered with a Teflon membrane¹⁶. In recent years, optical sensors have become a convenient alternative in several areas of research, including biological systems and organisms. Different principles and working mechanisms are used depending on the analyte to be measured²⁶ and for optical pH and CO₂ measurements, a combination of different fluorescent dyes which detect intensity changes in the time domain are used. Although optical based probes have been used to assess *in vivo* oxygen levels within the female reproductive tract of humans²⁷ and pigs²⁸, to our knowledge, this technology has never been used in reproductive organs for pH and CO₂ measurement.

The porcine species is being widely used as an experimental animal model in biomedicine because of its similarities with humans in many aspects (i.e. genomics and immunology system)^{29,30}. Despite the differences observed in the female genital tract when compared with humans, porcine arises as a good alternative to other commonly used animal models such as rodents. In addition, to produce these porcine biomodels in a standardized scale, Assisted Reproductive Technologies (ARTs) are necessary (reviewed by^{31,32}). However, porcine *in vitro* fertilization (IVF) is far from being as efficient as in other species as rodents or bovine³². The main problem is polyspermy (more than one spermatozoon penetrates the oocyte), which has been related with the protocols of gamete preparation, and the medium and ambient conditions established during the co-incubation of gametes, which makes the zygote non-viable. Thus, when the ambient parameters mimic the physiological values, as it was recently demonstrated by adjusting the oxygen level for IVF and embryo culture²⁸, porcine ARTs yield better results. With regard to the pH–CO₂–HCO₃⁻ levels, the sperm cells and oocytes used for IVF are directly immersed in a culture media whose values for these parameters might not be similar to the physiological. This is mainly due to the absence of appropriate knowledge about the real figures existing in the living animal. The same problem is found for the protocols of sperm preparation, since no consideration is taken towards the transitional period confronted by the sperm cells in their passage throughout the uterus. Given that there is an almost complete lack of knowledge about the regulatory mechanisms of uterine pH¹⁹, whether the uterine ambient, and particularly its luminal pH–CO₂–HCO₃⁻ levels are modulated by the presence of sperm is a topic which deserves further attention.

From the above information we hypothesized that the measurement of pH–CO₂–HCO₃[−] levels in the porcine uterus by minimally invasive methods and no medication will give reference results (physiological), which should help to better understand the physiological ambient of the uterus at different stages of the estrous cycle, and when measurements are taken after insemination, how these parameters are modulated by the presence of ejaculate. For this purpose we aimed the estimation of pH, CO₂ and HCO₃[−] under three different conditions, (i) sows in estrous before artificial insemination [E(−)AI]; (ii) sows in estrous 2 h after AI [E(+)AI] ; and (iii) sows in diestrous (non-estrous stage, [NE]). Ultimately, these results could be used to better adjust the in vitro protocols to increase the efficiency of porcine ARTs.

Results

Attempts to monitoring pH and CO₂ levels in the uterine cavity: validation of the technique. In general terms the technique used to approach the uterine lumen with the probes was successful. After implanting the insemination catheter in the cervix, the progress of the inner cannula up to 16 cm towards the uterine cavity helped to widen the distal portion of the cervical canal. Then, the successive approaches with the endovascular catheter -with temperature probe inside-, the pH and CO₂ probes were successful in nearly all the animals. One subjective but unequivocal sign of having reached the uterine cavity was the hand-feeling of a smooth progression of the probe after having overpassed the inner most cervical cushion. However, in two sows at NE condition, the deepest part of the cervical canal did not allow for further progress of the pH and CO₂ probes. On the other hand, CO₂ data from one pig at the three experimental situations were dismissed because inaccurate readings of the sensor, and from another pig at E(+)AI because of some bleeding was observed. Hence, the approach was successful in 68 out of 72 attempts (94.4%, pH and CO₂ probes in 12 animals at three different conditions) and the recording of data in 64 occasions (88.9%). The average insertion length of the pH and CO₂ probes was 64.2 ± 12.6 cm and 62.0 ± 11.5 cm, respectively. The average temperature of the uterus was 38.2 ± 0.3°C.

pH measurements within the uterus during estrous (before and after insemination) and diestrous stages. Individual plots of the timeline progress of all recorded pH values were represented (Fig. 1A–C). In some cases, the timeline depicted a waved pattern, with several variations higher than 0.2 pH units (Fig. 1A), while other displayed a quite a flat pattern with few and smooth waves (Fig. 1C). Characterization of flat and waved patterns was attempted depending on the number of waves higher than an established criterion, i.e. “a waved pattern displays > 2 waves of > 0.2 pH units”. However, as flat and waved patterns were found in animals of the three experimental conditions, and even within the same animal (i.e. Figure 1A, red and blue lines looked like a waved pattern while green line was rather flat) no statistical association between the defined pattern and the experimental conditions was found (Chi-squared test $p > 0.05$).

A comparison of the uterine pH with statistical significance between the three experimental conditions is shown in Fig. 2A. An immediate effect of AI on the uterus pH was dismissed. Although the average pH in E(−)AI sows was a bit lower than in the E(+)AI group (0.06 units), such a small difference was not found to be significant (7.05 ± 0.13 and 7.11 ± 0.16, respectively, $p > 0.05$). However, uterine pH in sows of the NE group (6.93 ± 0.16) was significantly lower than in groups E(−)AI and E(+)AI ($p = 0.017$ and $p = 0.007$, respectively), revealing a significant increase of the uterus pH at estrous.

CO₂ (%) levels within the uterus during estrous (before and after insemination) and diestrous stages. Plots of the timeline for the recorded % of CO₂ were obtained for each pig (Fig. 1D–F). As for the pH, the timeline variation of CO₂% described arbitrary curves with a variable number of undulations, indistinctly of the experimental group and individual.

A by-group comparison of the data with statistical significance is displayed in Fig. 2B. The average % of uterine CO₂ in E(−)AI group was 14.45 ± 3.58, and in E(+)AI group 13.12 ± 5.09. No effect of AI on the uterine % CO₂ was observed ($p > 0.05$), so the presence of sperm in the uterus for 2 h hardly changed the % CO₂. Conversely, the average uterine % CO₂ at NE condition was 8.44 ± 3.71, representing a very strong decrease between estrous and diestrous stages ($p_{NE vs E(-)AI} = 0.0002$ and $p_{NE vs E(+)AI} = 0.0019$).

HCO₃[−] concentration within the uterus in estrous (before and after insemination) and diestrous stages based on pH and CO₂ activity. The individual time progress of the estimated concentration of HCO₃[−] (mMol) was also represented in individual plots (Fig. 1G–I). As it was the case of pH and CO₂%, apparent flat or waved patterns were observed independently of the group and individual. As HCO₃[−] concentration was estimated by the Henderson-Hasselbalch equation with constant [CO₂%] (average value in each pig), bicarbonate variations resembled the pH pattern of the same animal in the same experimental condition. The average concentration of HCO₃[−] (mMol) was 35.16 ± 11.79 and 30.99 ± 14.21 (mMol) for E(−)AI and E(+)AI conditions, respectively. So, as it was found for pH and CO₂, AI did not change significantly the concentration of HCO₃[−] in the uterus ($p > 0.05$). Likewise, the average HCO₃[−] concentration in sows at NE condition was 14.94 ± 7.28 (mMol), which represented a high significant reduction with regards to the E(−)AI ($p = 0.001$) and E(+)AI ($p = 0.003$) (Fig. 2C).

Discussion

The in vivo characterization of the physiological ambient within the female reproductive organs of mammals is important for both basic studies in the field of reproductive biology and ARTs. For this reason, the knowledge of the physical and chemical properties of the reproductive milieu by in situ estimation or after fluid collection from the oviduct, uterus and vagina has been largely attempted in humans, livestock and animal models^{27,33}.

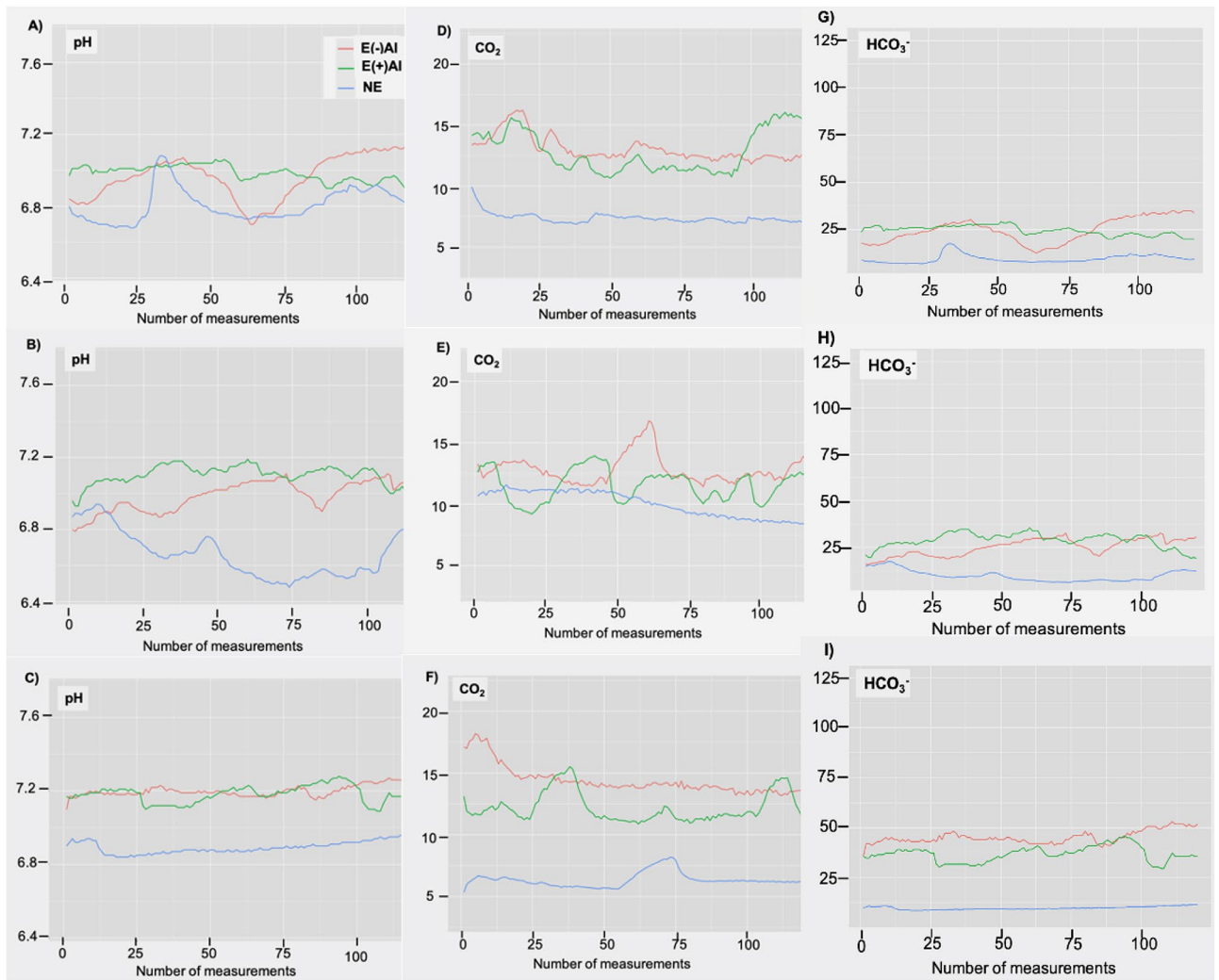


Figure 1. Plots of the timeline of uterine levels of pH (A–C), CO₂ (%) (D–F) and HCO₃⁻ (mMol) (G–I) at the three experimental conditions in three selected sows. Corresponding figures for the same animal are horizontally displayed. Lines are a continuous representation of a set of 120 individual measurements (1 dot was recorded every 5 s for 10 min).

With regards to the pH-CO₂-HCO₃⁻ biological regulator, very few studies have been addressed to the uterus (Table 1), which has only been approached in a few livestock species, but mainly cows. In pigs, only one work measured the uterine pH *in vivo* to a reduced number of animals via laparotomy¹⁷. In the present work, a more extensive study has been carried out and reference values of pH-CO₂-HCO₃⁻ levels in the porcine uterus have been obtained at two different stages of the estrous cycle and after insemination. Besides, to minimize iatrogenesis, all measurements were performed via a completely non-invasive cervical approach and without any medication. The combined use of a post-cervical AI catheter with miniaturized and semirigid pH and CO₂ dipping probes allowed successful approaches to the uterine cavity (body and horns) in more than 90% of the attempts. Although the method did not give a visual evidence of the position of the sensors inside the uterine cavity, it was validated by both the perception of a smooth progression of the probes after having crossed the cervical canal (subjective), and the figures of insertion length of the probes (objective). The latest criterium was verified by comparing our results with reported figures of the length of the reproductive tract -from the *rima vulvae* to the uterine cavity- measured in *ex-vivo* organs of multiparous sows³⁴. Our estimated insertion length for the pH and CO₂ probes -64.2 ± 12.6 cm and 62 ± 11.5 cm, respectively- was higher than the reported post-mortem length of those organs (56.25 ± 6.01 cm). On the other hand, the total absence of medication or animal distress during the procedure guaranteed that the measurements were the closest to the physiological values, as it was also the case when a similar post-cervical approach was carried out for estimation of uterine pH in cows^{20,21,25}.

Another novelty of the present work is the use of optical probes for the estimation of pH and CO₂. Up to now, virtually all *in vivo* measurements of pH in the female reproductive organs were based on a miniaturized pH glass electrode combined with a reference electrode. While this technology proved useful and accurate for pH estimation, that was not the situation for CO₂. Miniaturization of CO₂ potentiometric sensors is technically more limited because a Severinghaus-type electrode is built by covering the pH microelectrode with a cap and

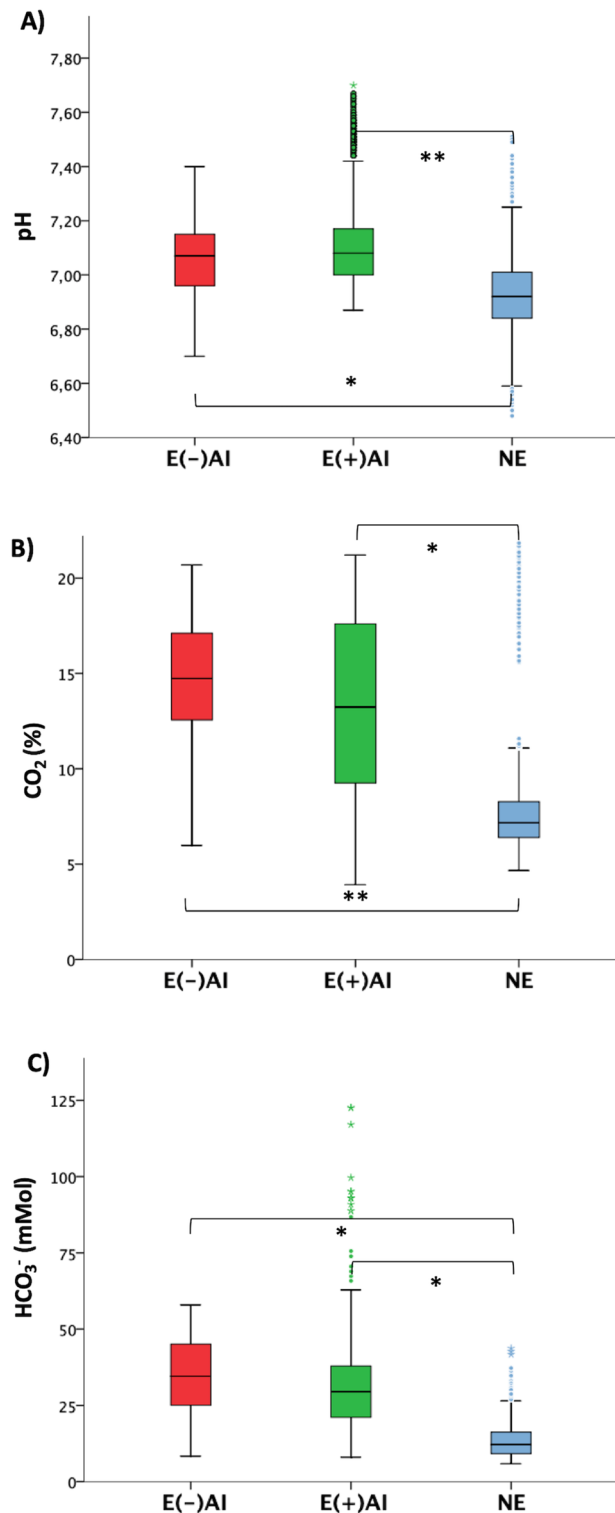


Figure 2. Box-plot describing the uterine levels of pH (A), CO₂ (%) (B) and HCO₃⁻ (mMol) (C) at the three experimental conditions. In each panel, significant differences between experimental groups are indicated as * $P < 0.05$ and ** $P < 0.001$. Small circles and asterisks in the box-plot represent outliers and extreme outliers, respectively.

a Teflon membrane in the tip. In addition to size restrictions for in vivo usage, the potentiometric CO₂ sensors have scattered readings when immersed in complex fluid matrix such as the oviductal or uterine fluids⁽¹⁶⁾; our experience with *unpublished results*). This most probably explains the lack of previous reports for CO₂ estimation in vivo (Table 1). Unlike the potentiometric sensors, luminescent (optical) probes can be miniaturized to a

few microns and display stable and accurate readings when immersed in complex biological matrix such as the reproductive fluids. This has already been proved for the determination of oxygen levels *in vivo* in reproductive organs of women and pigs^{27,28}. For the first time, this work describes how pH and CO₂ levels can be accurately measured in pigs with optical dipping probes directly inserted in the uterus. This is more relevant because from pH and CO₂ levels, HCO₃⁻ concentration was estimated, hence allowing a complete *in vivo* characterization of the bicarbonate buffer system. The relevance of characterizing this system for the optimization of ARTs protocols and the fact that the physical characteristics of the sensors (length, thickness, flexibility) can be customized under the manufacturer's advice envisages an increasing usage of this technology in other organs and/or species.

From the best of our knowledge, this is the first description of CO₂ and HCO₃⁻ levels measured *in situ* in the uterus of animals. Only one attempt has been previously described in the oviduct of primates¹⁶. In the case of pH, the information is more extensive; several studies have reported *in vivo* values either in oviduct or uterus in different species (see Table 1). In sows in estrous the estimated uterine pH was 6.98¹⁷, values slightly lower than those presented here (7.05–7.11). This fact might be related to the use of halothane anesthesia in the early report, because this anesthetic causes a slight decrease of pH, not only in the blood but also in the oviduct¹⁹. Another remarkable result obtained from the experiments was that pH, CO₂, and HCO₃⁻ levels were higher during the estrous stage, independently of the insemination condition, than during diestrous. It is worth noting that the levels of reproductive hormones are quite different at estrous and diestrous stages. While the estrous is mainly dominated by estrogens, the situation changes during the first days of diestrous, when progesterone levels start to raise (reviewed by³⁵). There are some evidences pointing that such hormonal changes have an impact on the pH-CO₂-HCO₃⁻ system. In rats, the expression of the different isoforms of carbonic anhydrase in the uterine tissue is directly regulated by the amount of estrogen and progesterone³⁶, which suggests that the physiological uterine levels of pH-CO₂-HCO₃⁻ are dynamic and likely modified by the precise hormonal levels at each stage of the estrous cycle. In fact, exogenous estrogens administered in ovariectomized rats caused an increase in uterine fluid secretion and HCO₃⁻ concentration³⁷. More recently, it has been proposed a secretion/reabsorption activity for HCO₃⁻ in the endometrial epithelium coordinated by progesterone and estrogen levels³⁸. In estrous stage, when estrogen is dominant, HCO₃⁻ secretion was up-regulated and reabsorption was down-regulated, and vice versa during diestrous stage, when progesterone is dominant. Nevertheless, the regulation of this complex system is far from being completely understood yet. For instance, different molecular mechanisms of the endometrial cells, such as the HCO₃⁻/Cl⁻ and Na⁺/HCO₃⁻/Cl⁻ exchangers, and the Na⁺/H⁺ antiporter are likely involved in the pH and HCO₃⁻ regulation³³. Also, CFTR (cystic fibrosis transmembrane conductance regulator), which is responsible of numerous secretory responses, is an active regulator of the uterine secretion of HCO₃⁻^{9,39}. CFTR expression is enhanced in the uterus of females in early estrous but not in other stages⁴¹ where progesterone is enhanced^{40,41}, suggesting a higher secretion of HCO₃⁻ during estrous which is in accordance with our observations. Hydration of CO₂ via carbonic anhydrase is another likely mechanism by which HCO₃⁻ accumulates in the uterine fluid¹⁴. Likewise, in the case of pH our findings are in agreement with the literature, where a more alkaline uterine environment is necessary for sperm transport, capacitation and fertilization³⁹ as occurs during the estrous stage, while an acidic pH, as observed in diestrous, is essential for embryo post-implantation development⁴².

Therefore, during estrous the uterine ambient is prepared to receive the spermatozoa, whose high levels of HCO₃⁻ and alkaline environment, among other components, are necessary to initiate the sperm capacitation, a essential process for fertilization. It has been previously demonstrated that semen deposition induces an increase in carbonic anhydrase activity in endometrial tissues⁴³ and causes a local shift in the gene expression of the female genital tract^{44,45}, including some genes involved in pH regulation⁴⁶. However, our results showed that the pH-CO₂-HCO₃⁻ system did not change after semen deposition within the uterus. Nevertheless, several factors could be involved in this fact. First, it has been shown that seminal plasma proteins and other components activated changes in the gene expression of the endometrium^{45,47,48}; but in our study, the level of seminal plasma in the insemination dose was low because the ejaculate was diluted in commercial extender, so its impact on the genomic expression of the endometrium could be limited. Second, although uterus is colonized by the sperm within minutes after deposition⁴⁹ the carbonic anhydrase activity in the endometrium is significantly increased after 4–6 h of sperm deposition⁴³, and differences detected in gene expression were evaluated 24 h after semen deposition^{44–46}; however, our measurements were carried out only 2 h after insemination. Thus, further studies with different time-points after insemination are necessary for a better understanding of the interactions between the uterine ambient and the sperm.

In vivo approaches to characterize the reproductive milieu such as the one in this study may have important benefits for the efficiency of the livestock industry and ARTs. Thus, the methodology presented here to estimate the levels of HCO₃⁻ within the uterus might be useful for the detection of animals with impaired secretion of this relevant molecule with regards to their particular pH and CO₂ values. Such alteration may result in compromised sperm capacitation, fertilization, or embryo development, as previously demonstrated in rodents^{50,51} and porcine⁵². So, having a criterion for assortment of sows with potential low fertility and/or prolificacy before entry to the reproductive system could be a relevant tool to optimizing the efficiency of the porcine industry⁵³. Consequently, further studies should be directed to investigate this technology applied to field conditions and this study also contributes to this field. On the other hand, ARTs are commonly based on mimicking physiological conditions. As an example, when the physiological O₂ tension found in the oviduct of sows was mimicked in the laboratory, both the final efficiency of embryo development and the quality increased compared to traditional conditions²⁸. However, despite some clear evidences found *in vitro*, little attention has been paid to mimicking the *in vivo* sperm transit, specially through the uterine horns, for further use in ARTs. Thus, pH, CO₂ or HCO₃⁻ variations in culture media during *in vitro* gamete preparation and/or interaction showed relevant consequences in the fertilization output^{54,55}. Also, an influence of the uterine fluid composition on sperm selection and consequent embryo culture was observed *in vitro* in several species including porcine^{56–58}. But, in spite of these findings, the current laboratory conditions of boar sperm preparation prior to IVF are yet quite standard in

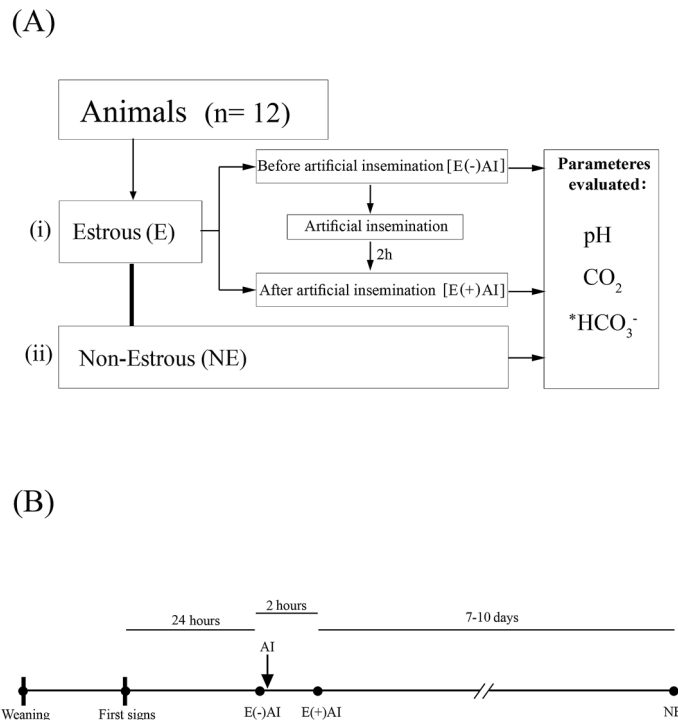


Figure 3. (A) Flow chart of the experimental design. HCO_3^- concentration was calculated from pH and CO_2 data. (B) Timeline of experimental design. Black dots indicate the moment when pH and CO_2 were measured.

the literature, using a temperature of 38.5°C , 5% CO_2 , 15–25 mMol of HCO_3^- and $\sim 7.2\text{--}7.4$ pH. This is relevant because such conditions are far from those observed in our study at the estrous stage, not only in their particular figures (except the temperature) but also in the fact that they are not steady but rather dynamic or even oscillatory. Therefore, as sperm preparation methods should be as physiological as possible³², more in vitro studies are required to test how sperm manipulation under closest to real physiological conditions have an impact on the fertilization output and subsequent embryo development. From the results of the present study and those for oxygen and temperature^{28,59} the optimal conditions for sperm preparation to be tested would be, temperature 38.5°C , pH $7.05\text{--}7.1$, CO_2 13–14%, HCO_3^- 30–35 mMol and O_2 7–10%.

Conclusions

This study shows for the first time the combined values of pH, CO_2 , and HCO_3^- in the uterus of the sow. By using a non-invasive approach and no medication during the procedure, precise reference values were obtained at different stages of the estrous cycle, and after insemination. The presence of sperm in the uterus hardly affected these parameters but were highly influenced by the estrous cycle, so that higher figures of pH, CO_2 , and HCO_3^- were found at estrous than at non-estrous stage (diestrous). The study contributes to (i) a better understanding of the in vivo regulation of the pH/ CO_2 / HCO_3^- system in the uterus; (ii) get reference values of pH, CO_2 , and HCO_3^- in a feasible physiological approach; (iii) obtain a new tool for tracking animals with impaired pH- CO_2 - HCO_3^- system; (iv) a new set of parameters for the in vitro media where sperm are treated before IVF, mimicking the physiological ambient.

Methods

Animals and study design. The experiment was conducted on 12 sows [*Landrace* \times commercial hybrids (*Large White* and *Duroc*) with 2.4 parities in average]. All animals showed normal reproductive performance previous to the experiments (total piglets born per litter 13.5 ± 1.0 ; live born per litter 10.9 ± 0.7). During the study, sows were housed in individual pens and fed by a commercial diet twice a day. Water was provided ad libitum. Environment was controlled by mechanical ventilation and evaporative cooling systems (temperature $22\text{--}24^\circ\text{C}$). Multiparous sows were weaned 28 days after farrowing. Thenceforth, sows were observed twice daily for estrous behavior. Those exhibiting vulva reddening and swelling, and a standing reflex were considered in estrous.

pH and CO_2 measurements were carried out at three different conditions (Fig. 3). The first set of measurements was done twenty-four hours after estrous detection [E(-)AI]. Immediately after this procedure, the females were artificially inseminated and 2 h later pH and CO_2 measurements were repeated [E(+)]AI. For the insemination, a single dose of diluted semen was used. The inclusion criteria to use the seminal doses was total motility $> 70\%$, viable sperm $> 90\%$ and morpho-abnormalities $\leq 15\%$. Post-cervical AI (seminal dose of 1.5×10^9 sperm/45 ml) was performed with a disposable catheter (Soft&Quick, TecnoVet S.L., Spain)⁶⁰. Finally, 7–10 days

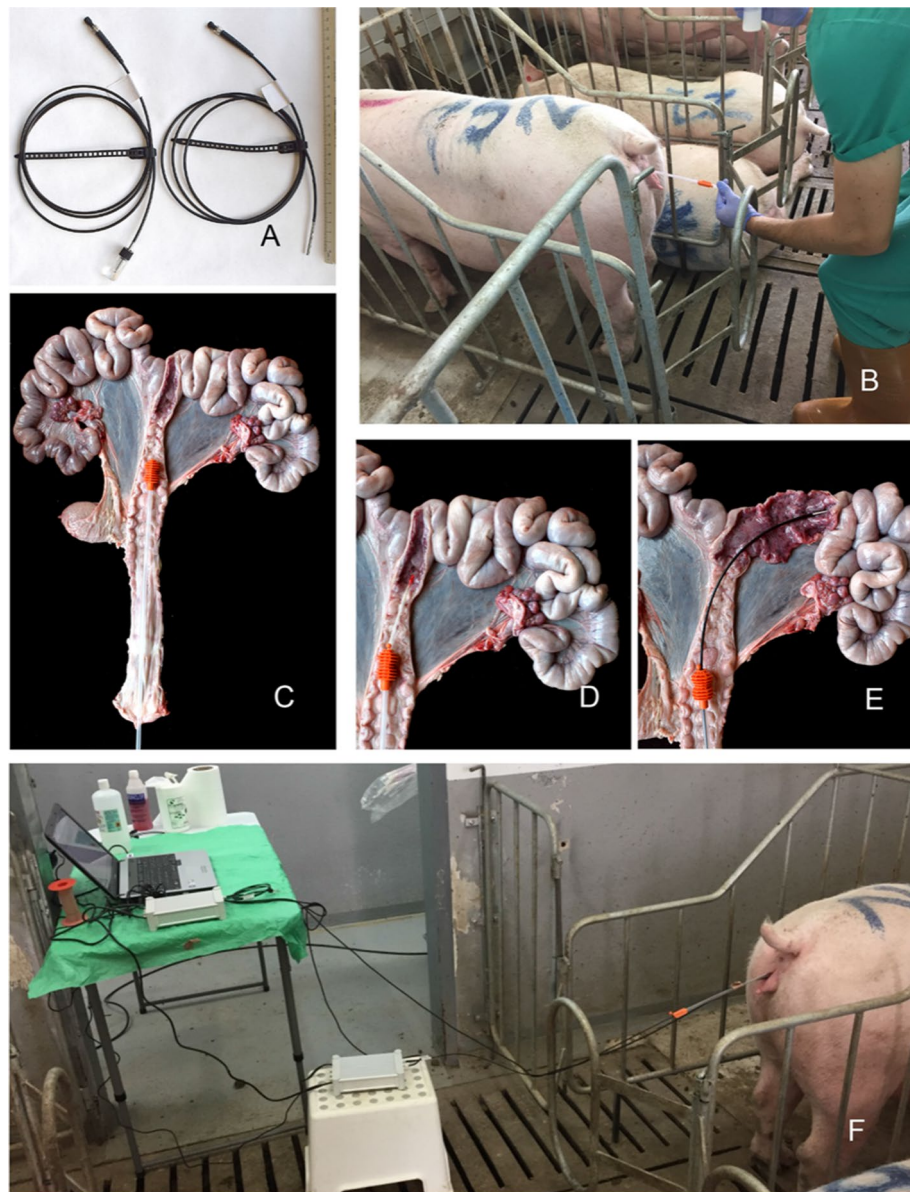


Figure 4. (A) Optical dipping probes of pH (right) and CO₂ (left). (B) Insertion of disposable post-cervical AI catheter. (C) *Ex-vivo* genital tract simulating the estimated location of the AI catheter in the cervix. (D) Detail of “C” showing how the inner cannula of the AI catheter reaches the body of the uterus. (E) Detail in *ex-vivo* reproductive tract simulating how the pH probe is inserted within the uterus. After having passed through the AI catheter, the probe was pushed up to reaching the body and horns of the uterus. (F) Data recording from the uterus once the stabilization period is over.

later, when the animals were at diestrous stage (non-estrous [NE]) the same procedure as at E(–)AI condition was repeated.

Measurement of pH and CO₂. pH and CO₂ optical dipping probes -1500 mm long and 3 mm thick were used (DP-HP5 and DP-CD1 prototypes, respectively, PreSens Precision Sensing GmbH—Am BioPark 11—93053 Regensburg, Germany) (Fig. 4A). Each probe was plugged into a recording unit (pH1 and PCO₂ mini, PreSens Precision Sensing GmbH), which was connected to a laptop. Probes were pre-calibrated by the manufacturer, but calibration was also checked the day before use with standard pH 7 solution (Crison Instruments S.A., Spain), or 5% CO₂ saturated saline solution (0.9%) within an embryo incubator. As pH and CO₂ values are influenced by temperature, uterine temperature was always measured before inserting the optical probes. This was done with a 1500 mm long and 0.5 mm thick thermistor connected to an amplifier device (ThermaData Logger TCD; E.T.I. Electronic Temperature Instruments, West Sussex, UK).

At the three sampling conditions the following routine was followed. Briefly, a disposable post-cervical catheter (Soft&Quick, TecnoVet S.L., Spain) was used to facilitate the approach to the uterine lumen (body and horns) with the probes (Fig. 4B). When the catheter was positioned in the cervix (Fig. 4C), the inner cannula was gently pushed cranially to “help to open” the cervical lumen (Fig. 4D). The inner cannula was then removed and replaced by an endovascular catheter -900 mm long and 2.92 mm inner diameter- (reference 54–89,001, Terumo Europe), which allowed the temperature probe to reach the uterine cavity. Temperature was recorded for 10 min, and the stable temperature value used as a reference for pH and CO₂ measurement in each animal. The endovascular catheter was then replaced by the pH probe, which was pushed cranially through the insemination catheter until no further progression was possible (Fig. 4E). When maximum insertion was reached, estimation of the insertion length within the reproductive tract was calculated by subtracting the total length of the probe to the distance from the *rima vulvae* to the recording unit. After a period of stabilization (2–5 min), and an indication to measure at the precise defined temperature, pH data were recorded every 5 s for a total of 10 min (Fig. 4F). The pH probe was then replaced by the CO₂ probe, and the same procedure was followed for the recording of CO₂ values. It is worth of mention that every time a probe was replaced the external opening of the insemination catheter was kept sealed to avoid the penetration of air within the cervix. All the protocol was done without any medication.

pH and CO₂ data were exported into a worksheet (Excel, Microsoft) and used to estimate HCO₃⁻ levels according to the Henderson-Hasselbalch equation, where pKa for a temperature of 38°C is 6.11. As a simultaneous record of pH and CO₂ was not available, the experimental average [CO₂] in each animal was set as a constant value in the equation, and a point by point variation of [HCO₃⁻] (mMol) was calculated depending on the pH.

$$[\text{HCO}_3^-] = [\text{CO}_2] \times 10^{(\text{pH}-\text{pKa})}$$

Statistical analysis. The statistical analysis was performed with the R program vs 3.4.4 (R Core Team 2018). Data of each experimental variable (pH, CO₂, HCO₃⁻) were explored by descriptive statistics and individual plots of the timeline of all the measurements displayed for each pig. ANOVA of repeated measures was carried out to evaluate potential differences between the three experimental conditions. Sphericity of data was always checked, and when required (as for the pH) corrected by Greenhouse–Geisser test. When ANOVA was significative between groups, comparison was done with post-hoc Tukey test. P values < 0.05 were considered statistically significant.

Ethics approval and consent to participate. All the procedures carried out in this work were approved by the Ethical Committee of Animal Experimentation of the University of Murcia and by the Animal Production Service of the Agriculture Department of the Region of Murcia (Spain) (Ref. N°. A13160606). Through the experiments, animals were handled carefully avoiding any unnecessary stress. All experiments were performed in accordance with relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines (<https://arriveguidelines.org/>).

Data availability

All data analyzed during this study are included in this article. Raw data used to build graphs and plots are available upon request to the authors.

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Author contributions

O.L.A. and F.A.G.V. are the project advisors, conceived and designed the experiments. O.L.A., P.J.L.L., R.L., F.A.G.V. performed the experiments and collected the data. O.L.A., JAO analyzed and interpreted data. O.L.A., F.A.G.V. wrote the draft of the paper. All authors reviewed the final version of the manuscript and gave final approval for the version to be published.

Competing interests

The authors declare no competing interests.

Additional information

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