In vivo measurement of pH, CO2 and HCO3- levels in the uterus of non-anesthetized sows through the estrous cycle and after insemination

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Research

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Abstract

Background

The pH-CO_2-HCO_3^- 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 Assistant Reproductive Techniques (ARTs). *In vivo* pH of the oviduct and uterus has been estimated by direct *in situ* measurements in a few species. However, regarding the levels of CO_2 and HCO_3^-, information is very scarce and, when available, it comes from fluid samples instead of *in vivo* estimations. This study describes a non-invasive method to measure pH and % of CO_2 in the uterus of sows with cutting-edge technology and no medication. Sows were at three different reproductive conditions, estrous with no insemination E(-)AI and after insemination E(+)AI, and diestrous (non-estrous, NE). From pH and CO_2 data, HCO_3^- concentration was estimated.

Results

The designed methodology allowed for *in situ* time-lapse recording of pH and % of CO_2 within the uterus of non-anesthetized sows. Variable oscillatory patterns of pH, CO_2 and HCO_3^- were found independently of the estrous condition. Insemination did not changed the levels of uterine pH, % of CO_2 and HCO_3^- concentration, -E(-)AI = E(+)AI-, but all the values were affected by the estrous cycle in a way that decreased significantly at diestrous condition - E(-)AI and E(+)AI > NE-.

Conclusions

A non-invasive approach to the porcine uterus with novel optical probes allowed the obtaining of *in situ* physiological values of pH, CO_2, and HCO_3^- at different reproductive conditions. While the short-time presence of sperm in the uterus did not change the physiological milieu, the whole pH-CO_2-HCO_3^- system was affected by the estrous cycle. This study contributes to a better understanding of the *in vivo* regulation of the pH/CO_2/HCO_3^- system in the uterus and may help to optimize the protocols of sperm treatment for *in vitro* fertilization.

Background

Carbon dioxide (CO_2) and H_2O 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 (H_2CO_3), which rapidly dissociates into a H^+ and a bicarbonate ion (HCO_3^-). The resulting equilibrium between CO_2, H^+ and HCO_3^- 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 HCO_3^- 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 HCO_3^- concentrations [3–5]. Both components are directly involved in many biochemical reactions resulting in the so called $sperm \cap acitation$, a process necessary for fertilization [6, 7]. HCO_3^- and pH play also an important role for embryo transport, development and implantation [2, 8]. The production and secretion of HCO_3^- 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, CO_2 and HCO_3^- 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 HCO_3^- 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 CO_2 tension (pCO_2) within the uterus or oviduct [14, 16]. In the porcine species, pH values in the oviduct are very variable (ranged from 6.7 to 8.3) depending on the study and the stage of the cycle [15, 17, 18], while HCO_3^- levels of 10.0-33.1 mMol were estimated in samples of oviductal fluid retrieved under surgical conditions [15]. In the uterus, pH (6.98) was only measured in a preliminary study in a series of 6 gilts [17], with no mention to CO_2 or HCO_3- values.

Table 1

Summary of the studies measuring pH, CO_2 and/or HCO_3^- levels directly in the female genital tract (uterus and/or oviduct) of different species.

Species	Organ	Estrous cycle	pH	HCO_3^- (mMol)	pCO_2	References
Rabbit	Oviduct	–	7.94	–	–	[24]
Primate (*Macaca mulatta)*	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]
	Oviduct	Post-ovulatory phase	7.37–7.40	–	–	[15]
		Estrous	6.7–8.3	–	–	

*calculated from CO_2 and pH values.

The characterization of physicochemical parameters *in vivo* is always a challenge. The use of medication

roach 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 mares [22], 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 pCO_2 measurements a pH electrode was converted to a Severinghaus type electrode covered with a Teflon membrane [16]. In recent years, optical sensors have become a convenient alternative in several application areas of research, including biological systems and organisms. Different principles and working mechanisms are used depending on the analyte to be measured [26] and for optical pH and CO_2 measurements, a combination of different fluorescent dyes which detect intensity changes in the time domain are used. Although optical based probes were used to assess *in vivo* oxygen levels within the female reproductive tract of humans [27] and pigs [28], to our knowledge, this technology has never been used in reproductive organs for pH and CO_2 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, assistant reproductive techniques (ARTs) are necessary [31, 32]. However, porcine *in vitro* fertilization (IVF) is far from being as efficient as in other species as rodents or bovine [32]. 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 stablished 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 culture [28], porcine ARTs yield better results. With regard to the pH-CO_2-HCO_3^- 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 pH [19], whether the uterine ambient, and particularly its luminal pH-CO_2-HCO_3^- 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-CO_2-HCO_3^- 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, CO_2 and HCO_3^- under three different conditions, i) sows in estrous before artificial insemination [E(-)AI]; ii) sows in estrous 2 hours 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.

Methods

Animals and study design

The experiment was conducted on 12 sows [*Landrace x* 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 22–24 ºC). 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. 1). 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 hours 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-abnomalies ≤ 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) [33]. Finally, 7–10 days 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 CO_2

pH and CO_2 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. 2A). Each probe was plugged into a recording unit (pH1 and PCO_2 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) and 5% CO_2 saturated medium solution (6.2%) within an embryo incubator. As pH and CO_2 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 (ThermaDataTM 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. 2B). When the catheter was positioned in the cervix (Fig. 2C), the inner cannula was gently pushed cranially to “help to open” the cervical lumen (Fig. 2D). The inner cannula was then removed and replaced by an endovascular catheter – 900 mm long and 2.92 mm inner diameter- (reference 54-89001, 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 CO_2 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. 2E). 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 seconds for a total of 10 min (Fig. 2F). The pH probe was then replaced by the CO_2 probe, and the same procedure was followed for the recording of CO_2 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 CO_2 data were exported into a worksheet (Excel®, Microsoft®) and used to estimate HCO_3^- levels according to the Henderson-Hasselbalch equation, where pKa for a temperature of 38 ºC is 6.11. As a simultaneous record of pH and CO_2 was not available, the experimental average $[CO_2]$ in each animal was set as a constant value in the equation, and a point by point variation of $[HCO_3^-]$ (mMol) was calculated depending on the pH.

$$[HCO_3^-] = [CO_2] * 10^{(pH - pKa)}$$

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, CO_2, HCO_3^-) 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.

Attempts to monitoring pH and CO_2 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 CO_2 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 CO_2 probes. On the other hand, CO_2 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 CO_2 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 CO_2 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 ºC.

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. 3A-C). In some cases, the timeline depicted waved pattern, with several variations higher than 0.2 pH units (Fig. 3A), while other displayed a quite a flat pattern with few and smooth waves (Fig. 3C). 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 3A, 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. 4. 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.

CO_2 (%) levels within the uterus during estrous (before and after insemination) and diestrous stages

Plots of the timeline for the recorded % of CO_2 were obtained for each pig (Fig. 3D-F). As for the pH, the timeline variation of CO_2 % 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. 4. The average % of uterine CO_2 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 % CO_2 was observed ($P > 0.05$), so the presence of sperm in the uterus for 2 hours hardly changed the % CO_2. Conversely, the average uterine % CO_2 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 $P_{NE\ vs\ E(+)AI} = 0.0019$).

HCO_3^- concentration within the uterus in estrous (before and after insemination) and diestrous stages based on pH and CO_2 activity

The individual time progress of the estimated concentration of HCO_3^- (mMol) was also represented in individual plots (Fig. 3G-I). As it was the case of pH and CO_2 %, apparent flat or waved patterns were observed independently of the group and individual. As HCO_3^- concentration was estimated by the Henderson-Hasselbalch equation with constant [CO_2 %] (average value in each pig), bicarbonate variations resembled the pH pattern of the same animal in the same experimental condition. The average concentration of HCO_3^- (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 CO_2, AI did not change significantly the concentration of HCO_3^- in the uterus ($P > 0.05$). Likewise, the average HCO_3^- 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 ($P = 0.003$).

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, 34]. With regards to the pH-CO_2-HCO_3^- 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 laparotomy [17]. In the present work, a more extensive study has been carried out and reference values of pH-CO_2-HCO_3^- 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 CO_2 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 sows [35]. Our estimated insertion length for the pH and CO_2 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 CO_2. 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 CO_2. Miniaturization of CO_2 potentiometric sensors is technically more limited because a Severinghaus-type electrode is built by covering the pH microelectrode with a cap and a Teflon membrane in the tip. In addition to size restrictions for *in vivo* usage, the potentiometric CO_2 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 CO_2 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 CO_2 levels can be accurately measured in pigs with optical dipping probes directly inserted in the uterus. This is more relevant because from pH and CO_2 levels, HCO_3^- 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.

One remarkable result obtained from the experiments was that pH, CO_2 and HCO_3^- levels were higher during the estrous stage independently of the insemination condition. 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 by [36]). There are some evidences pointing that such hormonal changes have an impact on the pH-CO_2-HCO_3^- 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 progesterone [37], which suggests that the physiological uterine levels of pH-CO_2-HCO_3^- are dynamic and likely modified by

the precise hormonal levels at each stage of the estrous cycle. More recently, it has been proposed a secretion/reabsorption activity for HCO_3^- in the endometrial epithelium coordinated by progesterone and estrogen levels [38]. In estrous stage, when estrogen is dominant, HCO_3^- secretion was up-regulated and reabsorption was down-regulated, and vice versa during diestrus 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 HCO_3^-/Cl^- and $Na^+/HCO_3^-/Cl^-$ exchangers, and the Na^+/H^+ antiporter are likely involved in the pH and HCO_3^- regulation [34]. Also, CFTR (cystic fibrosis transmembrane conductance regulator), which is responsible of numerous secretory responses, is an active regulator of the uterine secretion of HCO_3^- [9, 39]. CFTR expression is enhanced in the uterus of females in early estrous but not in other stages [11] where progesterone is enhanced [40, 41], suggesting a higher secretion of HCO_3^- during estrous which is in accordance with our observations. Hydration of CO_2 via carbonic anhydrase is another likely mechanism by which HCO_3^- accumulates in the uterine fluid [14]. 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 fertilization [39] as occurs during the estrous stage, while an acidic pH, as observed in diestrous, is essential for embryo post-implantation [42].

Therefore, during estrous the uterine ambient is prepared to receive the spermatozoa, whose high levels of HCO_3^- and alkaline environment, among other components, are necessary to initiate the sperm capacitation, a necessary process for fertilization. It has been previously demonstrated that semen deposition causes a local shift in the gene expression of the female genital tract [43, 44], including some genes involved in pH regulation [45]. However, our results showed that the pH-CO_2-HCO_3^- 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 [44, 46, 47]; 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 deposition [48] the differences detected in gene expression were evaluated 24 h after semen deposition [43–45], while our measurements were carried out only 2 hours 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 ARTs. It has been already mentioned that when the physiological O_2 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 conditions [28]. 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, CO_2 or HCO_3^- variations in culture

on showed relevant consequences in the

fertilization output [49, 50]. Also, an influence of the uterine fluid composition on sperm selection and consequent embryo culture was observed *in vitro* in several species including porcine [51–53]. But, in spite of these findings, the current laboratory conditions of boar sperm preparation prior to IVF are yet quite standard in the literature, using a temperature of 38.5 ºC, 5% CO_2, 15–25 mMol of HCO_3^- and ~ 7.2–7.4 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 possible [32], 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, 54] the optimal conditions for sperm preparation to be tested would be, temperature 38.5 ºC, pH 7.05–7.1, 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 a better understanding of the *in vivo* regulation of the pH-CO_2-HCO_3^- system in the uterus. Based on our findings, and trying to mimic the physiological ambient, a new set of parameters may be established for the *in vitro* media where sperm are treated prior to IVF.

Abbreviations

AI
Artificial insemination
ARTs
Assistant Reproductive Techniques
CO_2
Carbon dioxide
E
Estrous stage
HCO_3^-
Bicarbonate ion
H_2CO_3
Carbonic Acid
IVF

In Vitro Fertilization
NE
Non-estrous stage
PCAI
Post-cervical artificial insemination
pCO_2
Carbon dioxide tension

Declarations

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. Nº. A13160606). Through the experiments, animals were handled carefully avoiding any unnecessary stress.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declared that they have no competing interests.

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Author's contributions

OLA and FAGV are the project advisors, conceived and designed the experiments. OLA, PJLL, RL, FAGV performed the experiments and collected the data. OLA, JO analyzed and interpreted data. OLA, FAGV 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.

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Authors' information

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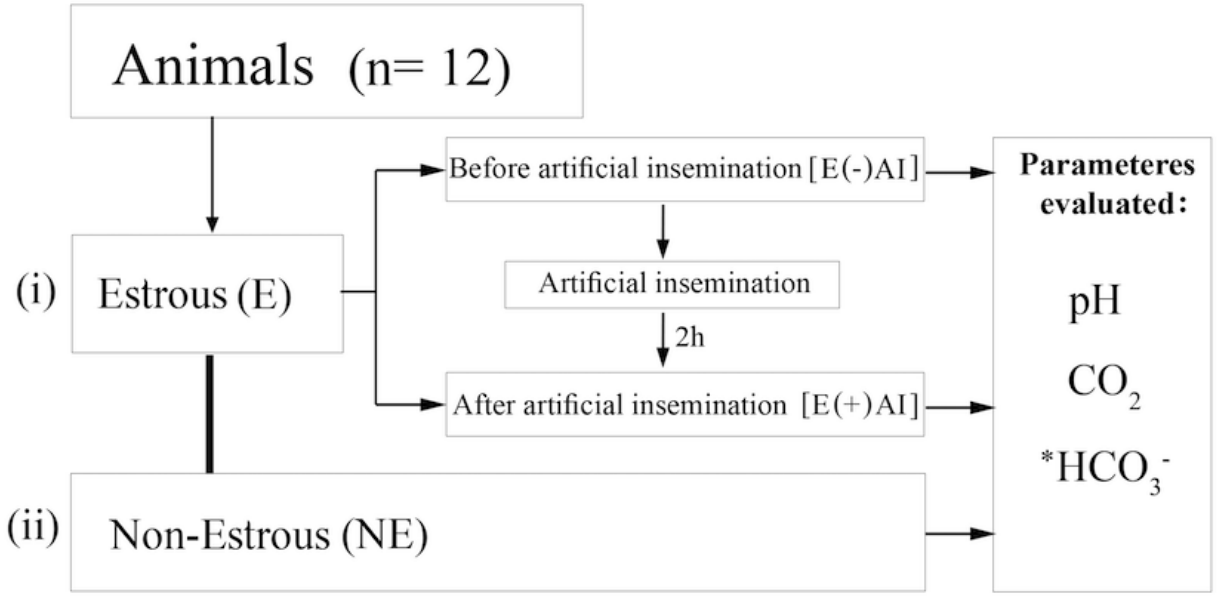
Figures

(A)



(B)

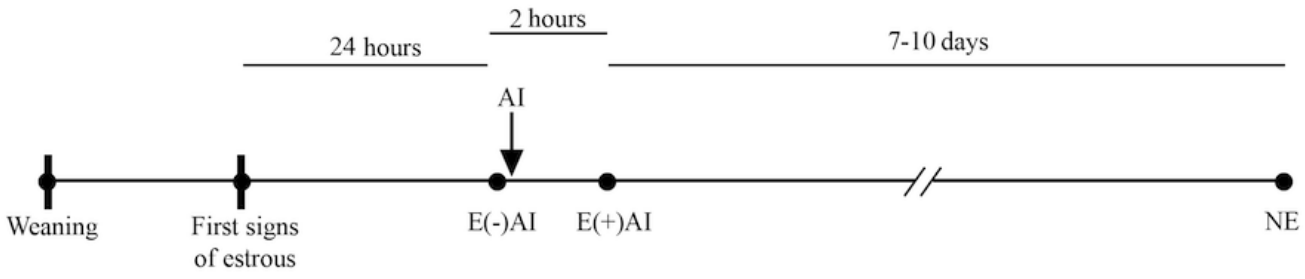


Figure 1

(A) Flow chart of the experimental design. *HCO3- concentration was calculated from pH and CO2 data.
(B) Timeline of experimental design. Black dots indicate the moment when pH and CO2 were measured.

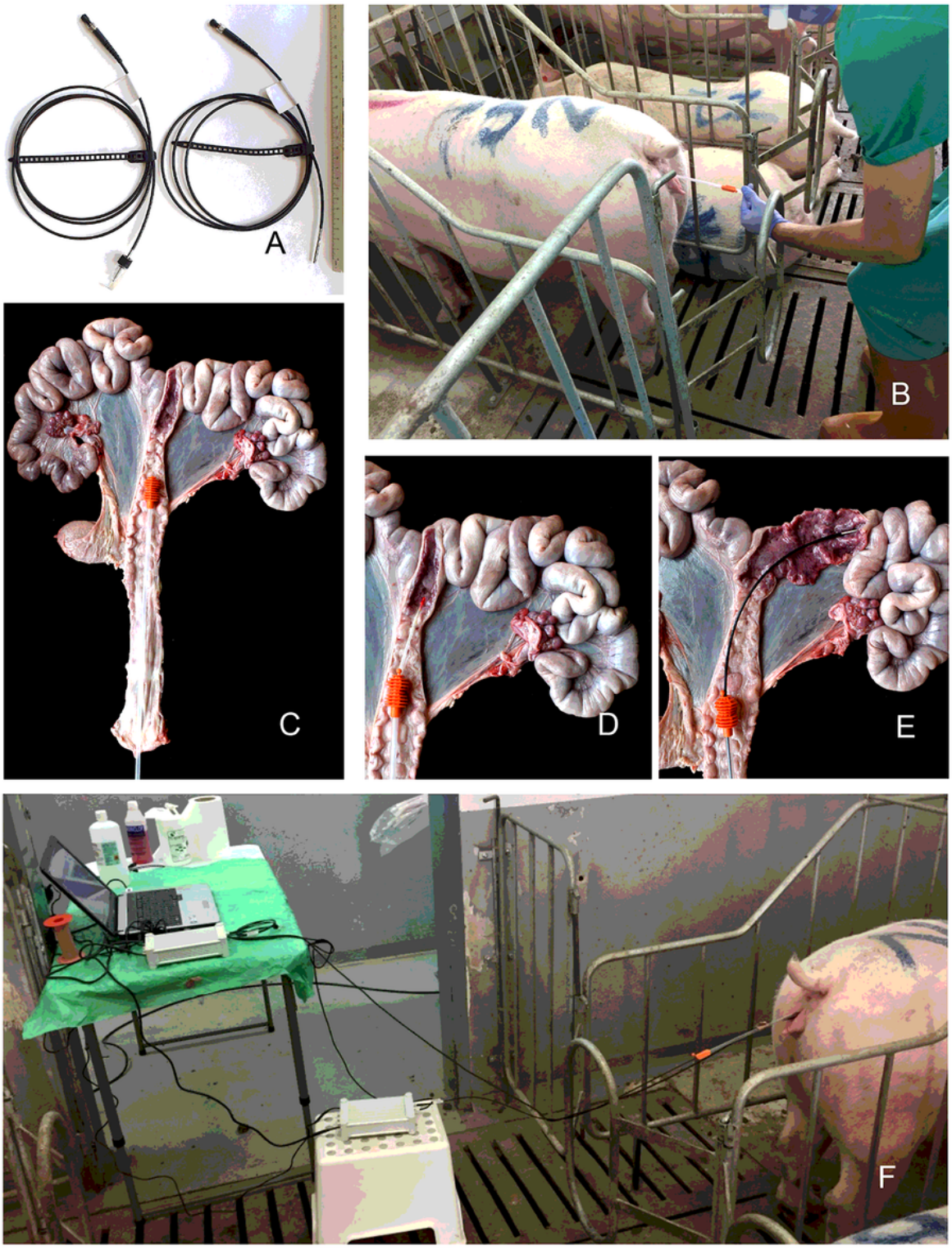


Figure 2

(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.

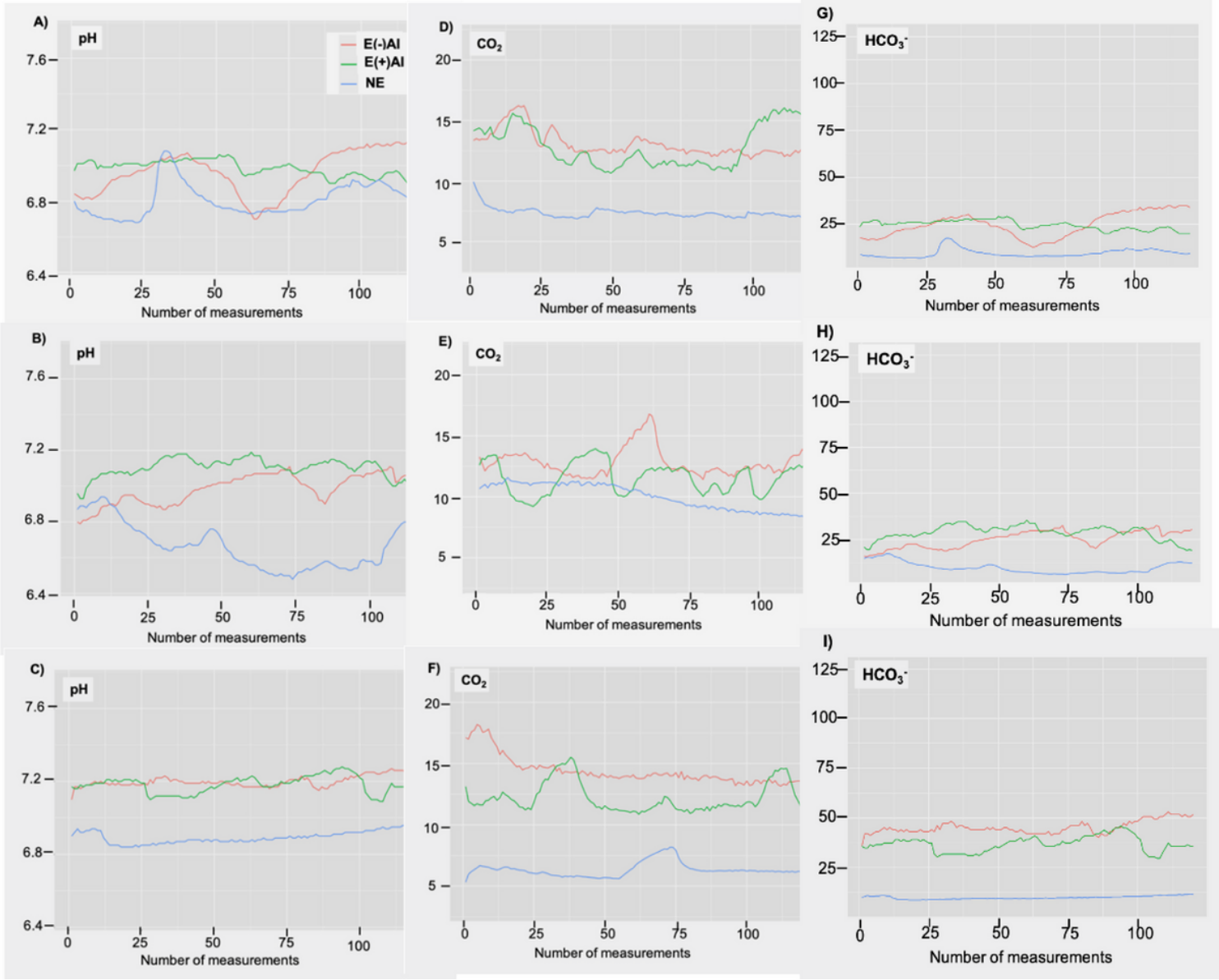


Figure 3

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 point was recorded every 5 seconds for 10 minutes).

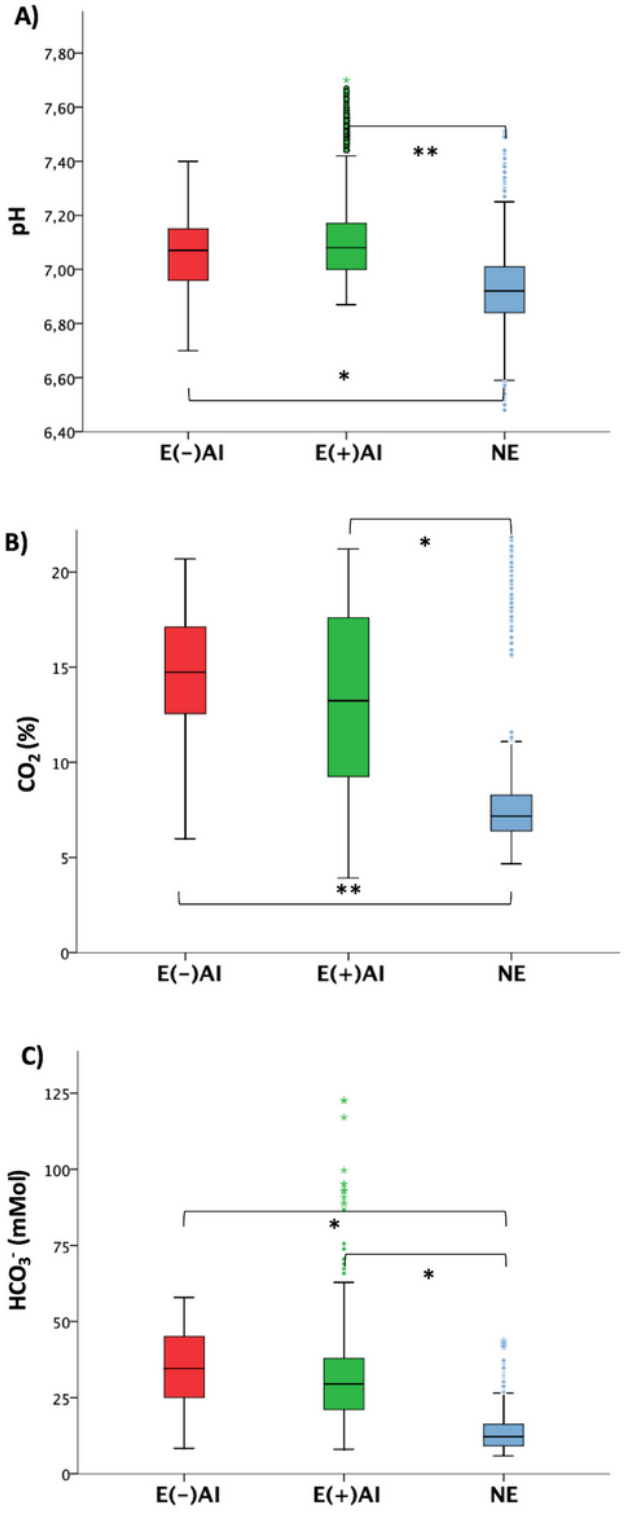


Figure 4

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.