Research in Veterinary Science 183 (2025) 105488

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Preliminary steps for fabrication of microfluidic systems for swine sperm sorting: Materials, perfusing systems and flow

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ARTICLE INFO

Keywords:
Microfluidics
Sperm sorting
Swine
Assisted reproductive technologies

ABSTRACT

The success rate of assisted reproductive techniques in the livestock production can be optimized by improving the quality of the semen sample by selecting only the good quality sperm from the ejaculate. Microfluidic technology has been studied for sperm sorting mainly in human ejaculates but has not been studied for boar sperm. Spermatozoa have been proven to be highly sensitive to different microplastics, but the potential toxic effects of the materials used to set up microfluidic systems have not been studied. The main goal of this study was to assess the possible toxic effect on boar sperm of materials commonly used for a microfluidic system and to evaluate the effect of different flow control systems (peristaltic pump, syringe pump and a microfluidic flow controller) at different flow rates (10 µl*min-1, 100 µl*min-1 and 1 ml*min-1) on sperm quality, as preliminary information for the development of a swine sperm sorting microfluidic system. Results showed no negative effect of the different materials at different concentrations. The control reached the highest curvilinear velocity compared to the peristaltic pump and the pressure-based flow control system. In the flow rates, 10 µm*min-1 showed the poorest results and no significant differences were observed between control and 1 mlmin-1 flow in any of the parameters. In conclusion, all materials that were studied for microfluidic fabrications were suitable for sperm sorting, any of the pumps would be suitable for sperm selection and 1 ml*min-1 flow rate would be the flow rate of choice for sperm pumping.

1. Introduction

Assisted reproductive technologies (ART), such as artificial insemination (AI), are widely used to increase reproductive rates, both for couples with fertility problems and in livestock production (Tournaye, 2000; Navarro-Rubio and Güell, 2020; Mellagi et al., 2023). Part of the success of ART is determined by semen quality. To control the success rate of ART in animal breeding, evaluation of semen quality is performed by determining semen characteristics such as sperm count, morphology and motility (Roldan, 2020; Abah et al., 2023). The success rate of ART in clinics and livestock production can be optimized by improving the quality of the semen sample by selecting only the good quality sperm from the ejaculate. Sperm selection is a widely researched technique, although so far there is no standard method for efficient sperm selection. Existing technologies for sperm sorting are mainly based on motility (swim up and gradient centrifugation) and apoptosis markers (MACS) (Xie et al., 2020). However, these techniques are time consuming and expensive, particularly for processing the substantial volumes of boar ejaculates. In addition to these commercial methodologies, researchers have paid increasing attention to mimicking the process of sperm sorting in the female reproductive tract, by integrating microfluidics as an alternative for sperm selection (Jahangiri et al., 2023).

Microfluidic technology has been studied for sperm sorting mainly in human ejaculates. Several microfluidic devices have been developed for sperm sorting over the past few years, but their efficacy remains to be optimized. Fertile Chip is the only commercial device tested in clinical studies but it did not improve fertilization in couples with unexplained infertility (Yetkinel et al., 2019). Microfluidic technology could also be useful for mimicking the female reproductive tract to enhance sperm sorting by combining these biomimetic models with orientation technics that occur in the female reproductive tract, such as rheotaxis,

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https://doi.org/10.1016/j.rvsc.2024.105488
Received 16 March 2024; Received in revised form 28 November 2024; Accepted 1 December 2024
Available online 3 December 2024

thermotaxis and chemotaxis (Zaferani et al., 2018; Ko et al., 2012; Nagata et al., 2018). These studies demonstrate the possibility of using microfluidic platforms to select spermatozoa according to their quality.

In terms of toxicity, spermatozoa have been proven to be highly sensitive to different drugs, nanoparticles or microplastics, affecting their motility, morphology or viability (Pérez-Duran et al., 2020; D'Angelo and Meccariello, 2021; Chen et al., 2023). Even though there have been several microfluidic systems designed to sort sperm, none of them have paid attention to the potential toxic effect of the materials used to set up these systems. The most common materials for fabricating microfluidic devices are PDMS (polydimethylsiloxane), PMMA (methyl methacrylate), COP/COC (cyclic olefin polymer /cyclic olefin copolymer) and PS (polystyrene), whereas for the tubing part of the system the most commonly used are silicon, FEP (Fluorinated Ethylene Propylene), PTFE (polytetrafluoroethylene) and PVC (polyvinyl chloride) (Gencturk et al., 2017; Ren et al., 2013; Van Midwoud et al., 2012). Materials used for the fabrication of devices have been widely tested for cell toxicity since they could affect cell viability depending on the cell type.

A crucial component of the microfluidic system is the flow control mechanism, which meticulously manages the perfusion of liquids in the circuit. This creates a specific level of shear stress that mirrors conditions within the female reproductive tract. There are different microfluidic flow control technologies commonly used in research such as rockers peristaltic pumps, perfusion-based systems and syringe pumps. The latter has been used for sperm sorting in different studies (Ahmadkhani et al., 2023; Eravuchira et al., 2018; Ghassemi Panah et al., 2022; Agarwal et al., 2016), but the effect of the others on sperm sorting remains unknown.

There are several publications on the use of microfluidic systems for sperm quality selection in humans. However, the use of microfluidic systems has been little explored in swine. The main goal of this study is to define the most suitable materials for the design of a microfluidic system and to evaluate the effect of the most commonly used flow control systems on sperm quality. This will provide preliminary and vital information for the development of a swine sperm sorting microfluidic system for high quality sperm selection.

2. Material and methods

2.1. Semen preparation

Three mature boars between 2 and 5 years old were selected for the study based on normal semen quality and proven fertility. All the boars were housed and fed according to animal welfare standards at a commercial boar semen collection unit (Semen Cardona stud, Tarazona, Spain). One ejaculate from each boar was collected manually by the gloved-hand technique. Only ejaculates with at least 70 % motile spermatozoa and 75 % morphologically normal spermatozoa were used. Semen was extended in Vitasem (Magapor, Zaragoza, Spain) to give a concentration of 30 million/ml spermatozoa and then cooled to +16 °C.

2.2. Experimental design

Experiment 1: Evaluation of toxicity of microdevice and tubing materials: Three different materials for microdevice fabrication (Polydimethylsiloxane [PDMS] (Sylgard Tm 184 Elastomer Kit), polymethyl methacrylate [PMMA] (Clear Acrylic Sheet Panel Model Number MCL0016), cyclo olefin polymer [COP] (ZeonorFilmTM ZF 14–188)) and three different tubing materials (polytetrafluoroethylene [PTFE] (Darwin microfluidics, LVF-KTU-15), fluorinated ethylene-propylene [FEP] (MFLX06406–60) and Tygon [Polyvinyl Chloride-based] (E-3603)) were tested. For each material, pieces of 1 cm × 1 cm were cut and added to a final sperm volume of 15 ml in different proportions (1, 5 or 10 pieces of each material). Sperm assessment was performed 24 and 72 h after incubation.

Experiment 2: Evaluate the effect of different flow control systems (peristaltic pump syringe pump and a microfluidic flow controller) at different flow rates (10 µl*min-1, 100 µl*min-1 and 1 ml*min-1) on sperm quality at four different times (5, 15, 30 min and 1 h): Three different systems for propelling sperm samples were assessed: peristaltic pump (Reglo Digital Pump, 4-Channel 12-Roller, Masterflex Ismatec), syringe pump (NE-1600 Six Channel Programmable Syringe Pump, Pump Systems Inc.) and a microfluidic flow controller (Flow EZ 1000 mbar, Fluigent) with a flow sensor (Flow unit M, Fluigent).

Peristaltic and perfusion systems were set up as follows: samples were placed in tubes (188–271, Cellstar tubes), which were connected with tubing to the peristaltic pump or the perfusion system. Once the sample started to flow through the system, it was collected in a final reservoir. For the syringe pump, samples were placed in a syringe (5200-000 V0, HENKE-JECT) and collected as previously mentioned. Three different flow rates (10 µl*min-1, 100 µl*min-1 and 1 ml*min-1) were assessed. Sperm assessment was performed at four different times (5, 15, 30 min and 1 h).

2.3. Sperm evaluation

Motility parameters: To determine sperm motility parameters, aliquots of semen samples (2 µl) were placed in a disposable chamber slide (Life optic slide, 20 µm depth) for analysis using Computer Assisted Sperm Analysis (CASA; ISAS system, Spain) at 37 °C under negative phase contrast microscopy and ×10 objective. Semen was diluted to obtain between 100 and 120 spermatozoa per field and four fields were analysed (approximately 400 sperm in total) at a frame rate of 30/s. Particles of size between 13 and 101 µm were considered spermatozoa. For all experiments total motility (TM), progressive motility (PM) and motion kinetics parameters were analysed with (CASA) and recorded. The kinetics parameters were as follows: VCL (Curvilinear displacement velocity; µm*s-1), VSL (Rectilinear displacement velocity; µm*s-1), VAP (Average trajectory velocity; µm*s-1), LIN (Linearity; %), STR (Straightness ratio; %), WOB (Oscillation ratio; %), ALH (Amplitude of lateral head displacement; µm) and BCF (Beat cross frequency, Hz). Setting for progressive motility was: STR > 70 % and VAP > 40 µm/s.

Evaluation of vitality and morphological abnormalities: Eosin-nigrosin staining was used to assess morphology and vitality (Bernard et al., 2019).One drop of the sample was mixed with one drop of stain and spread on a glass slide. The smear was dried and immediately observed under the microscope to assess the percentages of abnormal heads, mid- pieces and tails as well as the presence of droplets. A total of 200 sperm were evaluated and the percentage of abnormal sperm was calculated. In addition, spermatozoa were recorded to have an 'intact membrane' if not stained or to be 'dead' if stained. A total of 200 sperm were counted per sample and the percentage of viable sperm was calculated.

2.4. Statistics

Statistical analyses were performed using SPSS Statistics 19.0. General linear model (GLM) analysis with a post-hoc Duncan test was performed and interactions between factors were calculated. Statistical tests were considered as significant for P values below 0.05 ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Values are given as the means ± standard deviation.

3. Results

3.1. Experiment 1

Three materials for device fabrication (PDMS, PMMA, COP), three tubing materials (TYGON, PTFE, FEP), and three concentrations of each material were evaluated (Fig. 1). Samples were incubated with different materials and their effect on sperm parameters were evaluated after 24 h

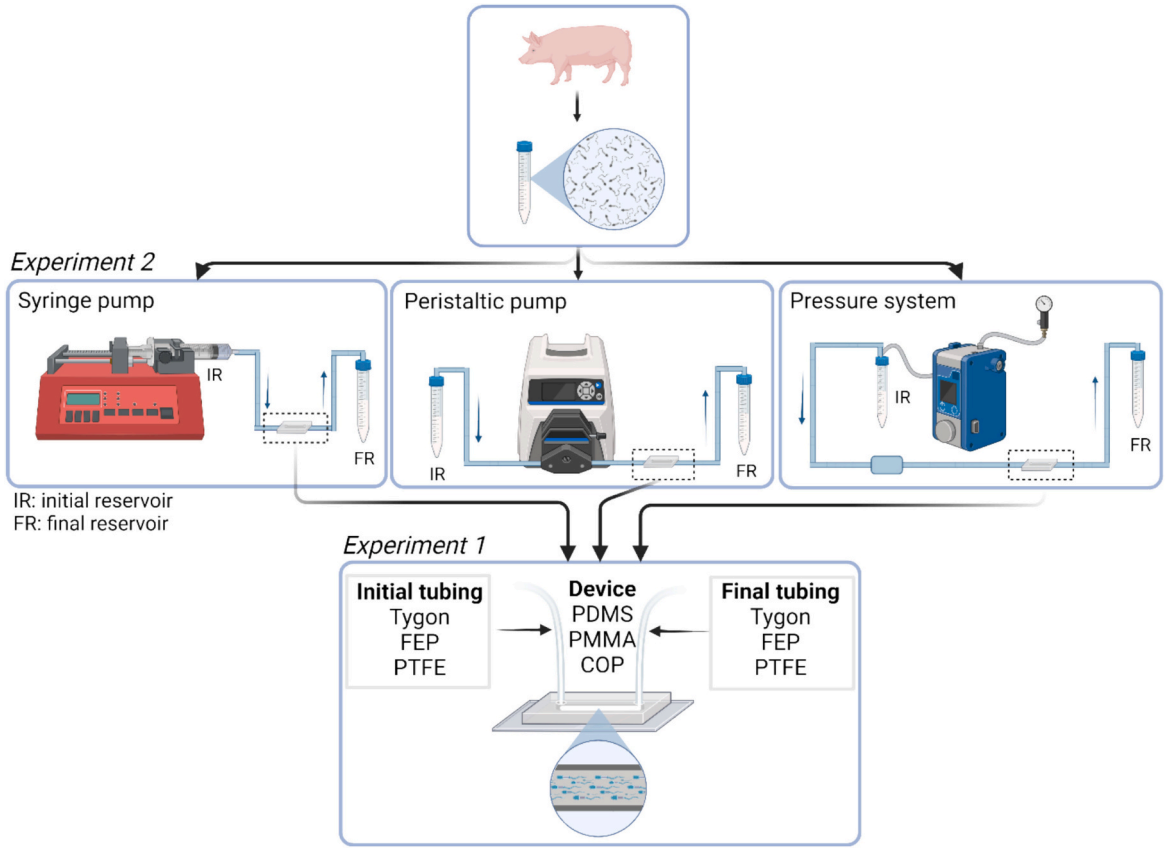

Fig. 1. Experimental design for testing materials and different microfluidic setups. Evaluation of toxicity to microdevices and tubing materials was performed in *Experiment 1*. PMDS, PMMA and COP were the materials tested for the fabrication of microfluidic devices and PVC (Tygon), FEP and PTFE for the tubing part of the setup. For the *Experiment 2* different microfluidic systems setups were tested for performing the assays with sperm samples flowing through the system at different fluid flow rates and at different times. Created with Biorender.

and 48 h of direct contact (Table 1). Results showed no negative effect of the different materials at different concentrations (Supplementary 1). At 24 h there was a negative effect of FEP on BCF parameter, but this disappeared after 48 h of incubation. (See Fig. 2.)

3.2. Experiment 2

Three different systems for applying flow were tested (peristaltic pump, syringe pump and pressure system) at different flow rates and also at for different time periods(Fig. 1). In terms of the system used for pumping the samples, significant differences were only observed in VCL, where the control gave a significantly higher values compared with the peristaltic pump and the pressure-based flow control system (Table 2). For the flow rates, no significant differences were observed between the control and 1 ml*min flow in any of the parameters, however the 100 µm*min-1 flow showed significantly lower values than the control in VIT, VAP and BCF and the 10 µm*min-1 flow rate gave the lowest values overall (Table 3). The TM, MP, VIT and MA were maintained within the analysis time. Significant pump and flow interactions were observed for TM (*p*-value 0.009) and VIT (p-value 0.003). A decrease was observed in TM and VIT using the 10 µm*min-1 flow rate in the pressure system and peristaltic pump compared with the other flow rates. No differences between flow rates were observed using the syringe pump. These results may be related to the mechanism of the systems themselves.

Finally, sperm samples were pumped through the systems for either 5 min, 15 min, 30 min or one hour, and no differences were found in parameters related to motility, vitality and morphological abnormalities (TM, PM, VIT and MA) (Fig. 3). In addition, there were no differences in any of the other parameters studied (VCL, VSL, LIN, STRE, WOB, ALH, BCF) for the different time periods (Supplementary 2).

4. Discussion

Selecting the appropriate material is the initial step in creating a microfluidic device, as the choice varies based on the specific requirements and the nature of the experiments to be conducted. Since work began on this type of technology applied to cell culture almost two decades ago, microfluidic chips have been made of various materials with different properties (Ren et al., 2013). Devices made from polymer-based materials are the most widely used in the field of microfluidics, as their surface is easily modifiable for biomedical applications and they are biocompatible with cell culture (Gencturk et al., 2017). To the best of our knowledge this is the first study comparing the effect of three materials for device fabrication (PDMS, PMMA, COP), three tubing materials (TYGON, PTFE, FEP), and three concentrations of each material on sperm quality. Any of the materials commonly used for microfluidic devices fabrication tested could be suitable for sperm processing. PDMS is the most widely used elastomer in microfluidics, partly because of its low cost and also offers the advantage of being a versatile material with which to make complex models and is gas permeable, which allows oxygenation of the culture media. However, it has some limitations since it is a hydrophobic material, which means that its surface has to be treated to be compatible with cell adhesion. It is a very porous material and therefore adsorption of small molecules and lipids from the culture medium to the PDMS occurs (Van Midwoud et al., 2012; Mehling and Tay, 2014). Thermoplastic polymers such PMMA and COP have also emerged in the field of microfluidics offering outstanding optical properties and a robust and scalable production method. These

Table 1
Effect of materials on sperm parameters after 24 h and 48 h incubation. Data are given as mean ± standard deviation. *P*-value: * < 0.05.

	Device								Tubing							
	Control		PDMS		PMMA		COP		PVC (Tygon)		PTFE		FEP		p-value	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
TM	49,00 ± 17,07	60,60 ± 1,50	47,11 ± 11,31	51,14 ± 10,74	38,12 ± 12,41	51,18 ± 10,15	47,87 ± 12,15	58,91 ± 7,92	47,68 ± 15,48	54,77 ± 9,08	54,70 ± 14,70	58,74 ± 9,77	52,45 ± 8,81	61,54 ± 6,07	0,117	0,14
PM	38,86 ± 14,97	41,10 ± 6,05	35,61 ± 8,91	34,97 ± 7,85	29,53 ± 11,03	34,37 ± 8,55	38,71 ± 10,86	42,15 ± 5,91	37,34 ± 13,01	38,72 ± 7,14	42,33 ± 13,40	38,98 ± 5,66	38,36 ± 6,00	41,065 ± 5,66	0,279	0,147
VCL	54,01 ± 6,94	87,78 ± 8,88	61,06 ± 11,81	85,34 ± 9,80	60,41 ± 9,74	87,34 ± 11,44	64,39 ± 7,81	90,21 ± 1057	6,47 ± 5,42	93,51 ± 11,78	58,57 ± 8,85	92,59 ± 7,23	55,86 ± 10,96	89,61 ± 10,44	0,599	0,653
VSL	29,18 ± 1,86	47,70 ± 7,00	31,65 ± 5,04	45,09 ± 4,48	29,18 ± 7,31	46,18 ± 7,85	34,91 ± 6,76	49,13 ± 3,79	32,25 ± 3,42	50,65 ± 6,66	31,67 ± 4,00	47,42 ± 6,37	29,39 ± 5,07	46,53 ± 5,74	0,328	0,41
VAP	36,86 ± 2,20	68,67 ± 3,20	41,13 ± 7,33	62,81 ± 6,23	38,37 ± 7,64	64,97 ± 7,23	43,39 ± 7,97	68,25 ± 6+,22	41,33 ± 3,78	68,97 ± 7,33	40,63 ± 6,12	70,08 ± 3,98	38,11 ± 7,56	68,83 ± 5,25	0,64	0,157
LIN	54,42 ± 5,25	54,41 ± 7,18	52,52 ± 6,66	53,20 ± 5,85	48,42 ± 9,01	52,74 ± 5,24	54,06 ± 6,89	57,74 ± 3,40	52,75 ± 6,71	54,21 ± 3,53	54,32 ± 3,72	51,32 ± 6,78	52,94 ± 3,95	52,23 ± 6,79	0,496	0,825
STR	79,26 ± 2,53	69,34 ± 8,64	77,30 ± 4,00	71,93 ± 4,81	75,62 ± 6,08	70,78 ± 7,13	80,37 ± 3,49	72,12 ± 4,09	77,97 ± 2,46	73,36 ± 4,79	78,21 ± 2,37	67,69 ± 8,31	77,49 ± 3,32	67,58 ± 6,63	0,276	0,312
WOB	68,58 ± 4,91	78,51 ± 4,13	67,75 ± 5,93	73,94 ± 6,34	63,56 ± 6,95	74,59 ± 3,34	67,11 ± 6,38	75,89 ± 3,38	67,52 ± 7,03	73,92 ± 2,13	69,47 ± 4,58	75,85 ± 3,62	68,30 ± 3,78	77,14 ± 3,85	0,437	0,607
ALH	2,36 ± 0,37	3167 ± 0,72	2,58 ± 0,47	3,41 ± 0,69	2,61 ± 0,37	3,37 ± 0,62	2,65 ± 0,28	3,42 ± 0,60	2,58 ± 0,26	3,71 ± 0,61	2,47 ± 0,36	3,47 ± 0,58	2,46 ± 0,36	3,36 ± 0,65	0,892	0,918
BCF	8,46 ± 0,50ab	7,93 ± 0,68	8,45 ± 0,49ab	7,60 ± 0,51	8,65 ± 0,45ab	7,70 ± 0,32	8,80 ± 0,37ab	7,76 ± 0,40	8,90 ± 0,42a	7,73 ± 0,50	8,57 ± 0,43ab	7,66 ± 0,54	8,31 ± 0,31b	7,56 ± 0,60	**0,041***	0,968
VIT	85,36 ± 4,33	86,24 ± 4,13	84,36 ± 4,22	85,17 ± 4,19	85,99 ± 3,23	84,99 ± 5,32	85,12 ± 4,12	85,21 ± 4,27	85,19 ± 4,22	85,36 ± 4,23	85,12 ± 4,15	85,27 ± 4,29	85,19 ± 4,19	85,89 ± 4,23	0,802	0,899
MA	22,33 ± 1,67	22,67 ± 1,23	22,19 ± 1.,2	21,23 ± 1,87	22,99 ± 1,56	22,56 ± 1,37	21,44 ± 1,02	23,34 ± 1,99	23,12 ± 1,55	22,31 ± 1,57	22,31 ± 1,99	22,56 ± 1,23	22,44 ± 1,02	21,34 ± 2,01	0,812	0,901

TM: total motility (%); PM: progressive motility (%); VCL: curvilinear velocity (microm/s); VSL: straight line velocity (microm/s); LIN: lineality (%); STR: straightness (%); WOB: wobble index (%); ALH: lateral head displacement (microm); BCF: beat cross frequency (Hz); VIT: vitality (%); morphological abnormalities (%).

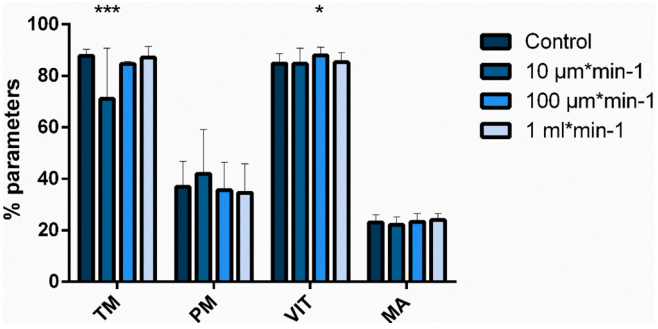

Fig. 2. Total motility (TM), progressive motility (PM), vitality (VIT) and morphological abnormalities (MA) were analysed applying different flow rates to the samples. Data are given as mean ± standard deviation. p-value: *** <0.001; * < 0.05.

materials present high transmittance similar to that of glass, low adsorption, are resistant to chemicals (even polar solvents), and have low autofluorescence. Their low production cost, because they can be manufactured in large batches by injection moulding, makes them a good material of choice for device manufacture (Bernard et al., 2019; Nunes et al., 2010). Several microfluidic devices have been designed using these materials for sperm monitoring and sorting and all of them appeared to be good candidates for microfluidic device fabrication (Yetkinel et al., 2019; Zaferani et al., 2018; Shirota et al., 2016; Quinn et al., 2018; Zhang et al., 2011). Our results corroborate these findings showing that all parameters studied were not affected by the different materials used for the fabrication of microfluidic devices. Apart from the device, the selection of tubing is also relevant for setting up the system, as it connects the different elements of the microfluidic circuit. In this

Table 2
Effect of the perfuse system on sperm parameters. Data are given as mean ± standard deviation. *p*-value: * < 0.05.

	Control	Syringe pump	Peristaltic pump	Pressure system	p-value
TM	87,78 ± 2,54	84,60 ± 4,34	80,73 ± 17,29	81,88 ± 13,94	0,125
PM	36,90 ± 9,91	35,37 ± 13,41	34,58 ± 11,68	41,22 ± 13,24	0,053
VCL	101,92 ± 4,48a	86,76 ± 17,39ab	83,05 ± 23,73b	81,71 ± 25,02b	**0,044***
VSL	35,98 ± 6,99	28,86 ± 7,91	28,29 ± 8,28	30,76 ± 9,50	0,986
VAP	81,68 ± 1,81	65,29 ± 18,67	63,71 ± 22,25	62,56 ± 23,22	0,131
LIN	35,50 ± 7,86	33,76 ± 9,25	35,19 ± 8,90	38,83 ± 9,95	0,057
STR	44,10 ± 9,03	46,04 ± 12,04	47,42 ± 12,67	52,23 ± 12,21	0,195
WOB	80,20 ± 2,27	74,08 ± 10,17	75,02 ± 8,89	74,75 ± 11,11	0,666
ALH	2,92 ± 0,34	2,65 ± 0,45	2,58 ± 0,56	2,54 ± 0,58	0,287
BCF	7,38 ± 0,65	8,10 ± 1,01	7,75 ± 0,64	7,85 ± 0,79	0,107
VIT	84,80 ± 3,83	86,37 ± 4,83	85,97 ± 5,62	85,71 ± 2,32	0,292
MA	23,80 ± 3,12	22,10 ± 2,12	23,15 ± 2,12	24,01 ± 3,56	0,899

TM: total motility (%); PM: progressive motility (%); VCL: curvilinear velocity (microm/s); VSL: straight line velocity (microm/s); LIN: lineality (%); STR: straightness (%); WOB: wobble index (%); ALH: lateral head displacement (microm); BCF: beat cross frequency (Hz); VIT: vitality (%); MA: morphological abnormalities (%).

Table 3
Effect of flow rate on sperm parameters. Data are given as mean ± standard deviation. p-value: *** <0.001; ** < 0.01; * < 0.05.

	Control	1 ml*min^{-1}	10 µl*min^{-1}	100 µl*min^{-1}	p-value
VCL	101,92 ± 4,48a	98,88 ± 13,96a	59,99 ± 17,80b	84,22 ± 17,95a	**<0,001*****
VSL	35,98 ± 6,99a	33,36 ± 6,19a	22,03 ± 6,09b	29,56 ± 8,95ab	**<0,001*****
VAP	81,68 ± 1,81a	78,93 ± 10,70ab	40,71 ± 13,65c	63,74 ± 19,91b	**<0,001*****
LIN	35,50 ± 7,89	34,60 ± 9,08	39,03 ± 12,67	34,88 ± 7,12	0,095
STR	44,10 ± 9,03b	42,82 ± 9,37b	58,13 ± 16,66a	47,64 ± 8,22ab	**<0,001*****
WOB	80,20 ± 2,27a	80,07 ± 4,91a	67,18 ± 8,17b	74,00 ± 11,28ab	**<0,001*****
ALH	2,92 ± 0,34a	2,79 ± 0,61a	2,19 ± 0,29b	2,64 ± 0,42ab	**<0,001*****
BCF	7,38 ± 0,65a	7,53 ± 0,61ab	7,96 ± 0,59ab	8,19 ± 0,99b	**0,002****

VCL: curvilinear velocity (microm/s); VSL: straight line velocity (microm/s); LIN: lineality (%); STR: straightness (%); WOB: wobble index (%); ALH: lateral head displacement (microm); BCF: beat cross frequency (Hz).

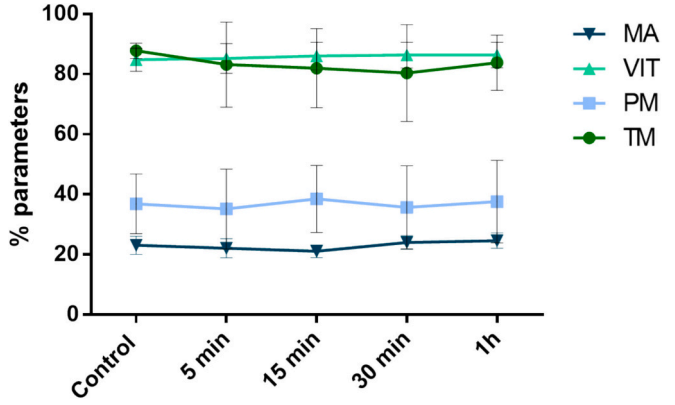

Fig. 3. Total motility (TM), progressive motility (PM), vitality (VIT) and morphological abnormalities (MA) were analysed at different times while applying fluid flow to the sperm samples.

study, different tubing materials (PVC, FEP and PTFE) were tested and as with the device materials, they did not show any negative effect. Eravuchira et al. set up a system using Tygon tubing, which is a PVC-based material, for individual sperm selection and they showed that by controlling the flow rate, it was possible to select sperm cells with normal morphological characteristics (Eravuchira et al., 2018). Most of the previous studies performed using microfluidic devices for sperm analysis were set under static conditions, which means that they did not use flow and therefore did not use tubing to perfuse the sperm samples into the microfluidic devices. Here we demonstrated that all type of tubing materials can be used for perfusing samples since they did not have a negative effect.

In this study, three different systems for applying flow were tested (peristaltic pump, syringe pump and pressure system) at different flow rates and also for different time periods. Limited information on flow systems and flows is available for sperm selection or analysis as previously mentioned most of the studies performed with microfluidic devices for sperm samples are carried out under static conditions. In the literature one of the most popular methods to apply a given flow rate over the channels of microfluidic devices is using the peristaltic pump to propel the culture medium through tubes connected to the device (Essig and Friedlander, 2003; Baudoin et al., 2007; Maggiorani et al., 2015). The flow generated is pulsatile, as the medium is propelled by rollers that press on the tubes, generating waves that make the flow not completely linear. The syringe pump is an easy-to-use, cheap tool and does not require specific additional equipment (Ross et al., 2021). Another way to apply flow to cell culture is to use pressure control systems that drive the culture medium by applying air at a controlled and constant perfusion within the reservoir. This also results in a linear rather than pulsatile flow. Pressure-based flow control systems are the only ones that offer continuous monitorization of the flow rate and any changes in pressure between reservoirs in the microfluidic circuit. On the other hand, these systems can cause problems due to the aforementioned sensor, not only because it generates heat in specific areas for measurements, but also because it can get blocked by the sample depending on its viscosity. The syringe pump has been the tool of choice for controlling the flow rate of sperm, when performing experiments using different injection rates (Zaferani et al., 2018; Eravuchira et al., 2018; Ghassemi Panah et al., 2022). Our results showed that the parameters studied did not vary independently of the flow tool used. Therefore, in addition to syringe pumps, the peristaltic pump and the perfusion system are tools that can be used for this purpose. A relevant factor to consider when using microfluidic technology is the shear stress applied to the semen sample. This stimulus is defined as the tangential force exerted on an area by a fluid flowing parallel to a surface. For that reason, shear stress generated in the tubing, and also in the microfluidic device, must be addressed (Hamacher et al., 2020). In this preliminary study of different materials used for setting up a microfluidic system, the effect of the shear stress was not studied. In future investigations, an in-depth study of the effect of shear stress on spermatozoa will be carried out. Our results showed that fluid flow rates in microfluidic devices used for sperm assays can affect sperm quality reaching 1 ml*min. Besides 10 µm*min-1 flow rate in the pressure system and peristaltic pump reduced sperm quality compared with the other flow rates. These results may be related to the mechanism of the systems themselves. The peristaltic pump consists of rollers that cause the liquid to circulate by the pressure of the tubes against the rollers. In addition, this mechanism can create backpressure with the passage of each roller, causing the liquid to circulate backwards and forwards. These effects are increased when using low flow rates such as the one used in these experiments (10 µl*min-1). In the case of pressure-based flow control systems, the critical point is the sensor that is used to control the flow. These sensors calculate the flow by heating the sample as it passes through them. If the sample is circulating at a low flow rate, the damage will potentially be greater since the spermatozoa will be exposed to a higher temperature as it passes through the sensor. For these reasons, the syringe pump would be the system of choice for the assembly of a microfluidic system.

Sperm samples were pumped through the systems for either 5 min, 15 min, 30 min or one hour, and no differences were found in sperm quality. These results are important since the volume of swine ejaculate is usually high and for that reason sperm sorting is expected to take longer periods of time than with other sperm samples such as human spermatozoa.

5. Conclusions

The present study showed that materials commonly used to make chips (PDMS, PMMA, COP) and tubing (TYGON, PTFE, FEP) have no toxic effects on spermatozoa. Moreover, syringe, peristaltic and perfusion pumps are suitable for sperm pumping. The syringe pump gave the best results for all three flow rates, whereas peristaltic pump and pressure-based flow control system presented significantly lower VCL at 10 µl*min-1. A flow rate of 1 ml*min-1 would be the flow rate of choice for sperm pumping due to the large volume of a porcine ejaculate, thus favoring rapid sample processing, reducing processing time and avoiding sample damage. All these steps are essential to develop a microfluidic device together with a microfluidic system suitable for swine sperm sorting. In addition, further studies are needed to focus on the design and validation of new microfluidic technology for handling

sperm samples.

Funding

PORCINNOVA PILOT project within the framework of the Rural Development Program for Aragon 2014–2020, co-financed by the European Union and the Government of Aragon.

CRediT authorship contribution statement

A. Lacueva-Aparicio: Writing – original draft, Methodology, Investigation. **R. Monge:** Supervision, Methodology, Funding acquisition, Conceptualization. **L. Serrano:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **C. Malo:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

Rosa Monge and Luis Serrano are partners of the company BEOnChip (Zaragoza, Spain). There is no conflict of interest in the other authors.

Acknowledgements

The authors are thankful to Semen Cardona for providing semen samples. Authors would like to thank the Area of Reproduction and Obstetrics from the Veterinary Faculty from the University of Zaragoza for their help with the CASA assessments and Dr. J.A. Skidmore for her help with English grammar corrections.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105488.

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