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Effect of Newtonian and shear thinning medium on human sperm motion within a microchannel

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ABSTRACT
Male infertility related issues are growing at a rapid pace. The swimming behaviour of sperm cells and their motility is important in achieving effective fertilization. The viscosity of the surrounding fluid plays a crucial role for sperm migration towards successful fertilization. Herein, we conduct experiments to study the motion of human sperm in Newtonian and non-Newtonian fluids of varying viscosity within a microchannel. We selected Agarose (AG), Acacia (GA) and Methyl Cellulose (MC) as solutes diluted in Phosphate-buffered Saline (PBS) to create a viscous environment. The quantified results of sperm motion in these fluids are reported in terms of velocity, motility, and amplitude. Sperm cells possess the highest average curvilinear velocity in AG solutions and the highest average straight-line velocity in MC solutions. The highest motility of sperm cells is observed in MC solution.

Keywords: Microchannel, motility, sperm cells, velocity, viscosity.

1. INTRODUCTION
Human infertility is a global health issue growing at a rapid pace in the 21st century affecting 8-12 % of couples. The majority of male infertility issues are associated with low count & quality of sperm, morphology and a low percentage of motile sperm [1]. Motility is considered to be a major factor responsible for successful fertilization. Sperm cells can be classified as Progressive, non-progressive and non-motile depending on their flow behaviour. Assisted reproductive technologies (ARTs) such as IVF (In vitro fertilization), IUI (Intrauterine insemination) and ICSI (intracytoplasmic sperm injection) are promising techniques to address infertility issues. These methods make use of conventional techniques like swim-up and density centrifugation to sort high-quality sperm. However, the sperm cells sorted using these techniques often suffer with low yield, high DNA fragmentation, and generation of Reactive oxygen species. The drawbacks associated with methods can be effectively addressed using microfluidics technology. This technology makes use of low sample volumes and closely resembles the natural selection mechanism involved in the fertilization process [2-5].

2. LITERATURE REVIEW AND OBJECTIVE
The sperm cell is divided into three basic parts: head, mid-piece and tail. The head containing a nucleus is flat, disc-shaped having ~5 µm in size. The tail is ~50 µm long and is responsible for the propulsion of the cell [6]. The mid-piece connecting the head and tail consists of mitochondria which provide motility to the cell. Human sperm has to migrate into a female reproductive tract in a highly viscous environment. The ability of a sperm to migrate in a viscous environment is a key parameter towards the successful fertilization of the egg. The migration of cells in cervical mucus involves movement in different viscous layers leading them to oocyte for successful fertilization. Kirkman et al. [7] showed that though viscous forces resist the motion of sperm in all viscous layers, still they can migrate with finite progression. Chen et al. [8] used a microfluidic device and reported that the sperm which can travel through highly viscous layers of cervical mucus show high motility and can fertilize the egg. Suarez et al. [9] studied the sperm motion in the female reproductive tract. They report that the cervical mucus contains high viscosity glycoproteins (mucins) and only a few motile and morphologically normal sperm (i.e., less than 1 %) were able to move towards the female reproductive tract. Stehnach et al. [10] showed that, due to the viscosity gradient in cervical mucus, the swimming speed of the cells reduces and they accumulate in the high viscosity regions. Schmoll et al. [11] demonstrated the spatial and temporal variation of ovarian fluid viscosity greatly affects the swimming pattern of motile sperm to fertilize eggs in different viscous environments. Nosrati et al. [12] reported that the swimming behaviour of sperm depends on the chemical, physiological and rheological respondents that are present in the female reproductive tract. Tung et al. [13] studied the motion of the sperm in viscoelastic fluids in which sperm forms a cluster of different sizes. Hyakutake et al. [14,15] showed that Newtonian and non-Newtonian characteristics of viscous fluids also affect the motility and velocity of sperm. Recently, many researchers have worked to study the motion characteristics of

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sperm in polymers like methylcellulose [16-18] and polyacrylamide solutions [19].
Our survey reveals that limited studies have been carried out to investigate the effect of viscous environment on motility and velocity of sperm cell motion. Exploiting sperm behaviour in different fluid medium may assist in separating and isolating the best swimmers for the ART. In this study, an attempt has been made to explore the behaviour of human sperm cells in Newtonian and non-Newtonian fluids of varying viscosity within a microchannel. To the best of our knowledge, nobody has used AG and GA to study sperm motion within a microchannel. The comparative behaviour of sperm motion in AG, GA and MC solutions in terms of velocity, motility and amplitudes are also investigated. The insights gained from this study can be further used to separate high-quality sperm.

3. MATERIALS AND METHODS

3.1 Design and fabrication

A design of a simple straight microchannel of a rectangular cross-section was prepared using AUTOCAD as shown in Fig. 1(a). The microchannel consists of single inlet-outlet reservoirs. The channel width and height was kept as 200 µm and 50 µm respectively. The fabrication of the channel was carried out using photolithography and soft lithography techniques. A negative photoresist (SU-8 2050) was dispensed on the silicon wafer and rotated at 3000 RPM to obtain a uniform thickness of 50 µm. The photoresist was prebaked before placing it in the mask aligner and was exposed to a UV lamp. Due to UV exposure, the unexposed portion of the photomask was washed out using the developer solution. Finally, the silicon SU-8 mold was post-baked.

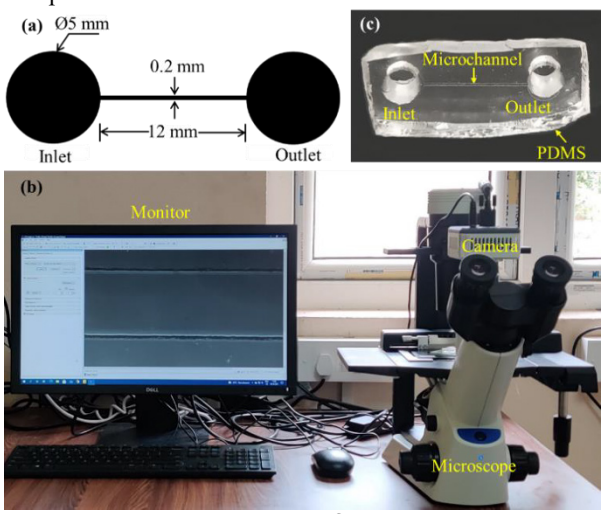

Figure 1: (a) Mask design of a straight microchannel (b) Experimental setup consisting of an inverted microscope, camera and a monitor and (c) Photograph of the PDMS based microchannel.

Further, employing soft lithography technique, a 10:1 (w/w) mixture of PDMS (Polydimethylsiloxane) and a curing agent was prepared and was poured on the silicon mold. The mold was then placed in a hot air oven at 65˚C for 45 minutes. After curing, PDMS was then peeled off from the mold and holes were drilled at inlet and outlet reservoirs. The fabricated PDMS chip was then bonded to the glass slide using an adhesive formed by a mixture of PDMS and curing agent in the 6:1 (w/w) ratio. To increase the bonding strength, the bonded PDMS chip was further heated in a hot air oven at 80˚C for 1 hour.

3.2 Sample preparation

Semen samples were collected from healthy humans (age 20-30 years with no previous history of infertility issues) in a wide-mouthed sterile container. The sample was allowed to liquefy for 30 minutes at 37˚C as per the WHO norms [20]. The viscosity of the solutions was changed by adding different concentrations of AG (LE Quick Dissolve Agarose, Genetix Biotech Asia Pvt. Ltd., India), GA (Acacia Extra Pure, Loba Chemie Pvt. Ltd, India), and MC (Methyl Cellulose 4000, Loba Chemie Pvt. Ltd., India) in PBS (Sigma-Aldrich, USA). MC is used as it is extensively used as surrounding fluid with sperm in the published literature [14-18]. AG and GA have shown promising results as sperm storage additives and hence they are well suited to study their effect on sperm flow behaviour [21,22]. The concentrations of polymers in PBS were 0.1% and 0.12% for AG, 10% and 12% for GA and 0.2%, 0.3% and 0.4% for MC as per w/v ratio. These polymer concentrations were chosen such that their solutions in PBS offers comparable viscosity with a Newtonian to non-Newtonian rheological transition. The experiments were conducted by diluting the liquefied semen in the ratio of 1:2 (v/v) in different solutions. The pH of all the solutions was maintained within the range of 7-7.5 to ensure the viability of sperm cells at a temperature of 37˚C.

3.3 Experimental setup and procedure

The experimental setup is shown in Fig. 1(b). It consists of an inverted microscope (CKX53, Olympus) with a digital camera (C11440-36U, ORCA-spark Hamamatsu) connected to it. The photograph of the PDMS based microchannel is shown in Fig. 1(c). A drop (10 µl) of semen sample diluted in different concentrations of AG, GA and MC was placed into the inlet reservoir of the microchannel and the cells were allowed to flow with their own velocity. Due to this, a finite volume gravity driven flow takes place in a microchannel. The effect of these viscous solutions on sperm velocity, motility and their amplitude were observed and recorded using HCImage Live software.

3.4 Velocity, motility and viscosity measurement

The kinematic parameters of sperm cells were characterized based on their velocity; curvilinear velocity (VCL), straight-line velocity (VSL) and Linearity. VCL is the actual distance travelled by the sperm cell over the time measured as a point-to-point track followed by the sperm cell whereas VSL is the straight distance travelled by the sperm cell over time from their initial to final positions. Velocities of around 80 sperm cells were measured using ImageJ software. Linearity and amplitude calculations provide information on the behaviour of progression of sperm cells. Linearity is the ratio of straight-line velocity to curvilinear velocity (VSL/VCL). The amplitude of the sperm trajectory (A) is defined as half the distance between

the two farthest points of both sides of the progress axis (refer Fig. 8).

Fig. 2 shows the motility classification of sperm cells based on VSL in different viscous solutions. The motility of sperm cells can be classified as immotile and non-progressive (0<VSL<5μm/s), slow-progressive (5<VSL<25μm/s) and rapid progressive (VSL>25μm/s).

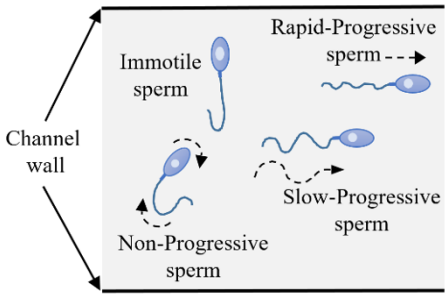

Figure 2: Schematic showing motility classification of sperm cells based on VSL. Rapid progressive (VSL>25μm/s), slow-progressive (5<VSL<25μm/s) and immotile or non-progressive (0<VSL<5μm/s).

The viscosity (μ) of solutions was measured using a Brookfield viscometer (DV2T) at 37°C. Moreover, the type of fluid (i.e., Newtonian or non-Newtonian) was determined by plotting the characteristic values of viscosity obtained under different shear rates (γ) as shown in Fig. 3. The shear rate is varied by changing spindle speed of the viscometer. The viscosity values and behaviour of fluids, average velocities and motility of cells are mentioned in Table 1. It can be observed from Fig. 3 that, the 0.1% AG, 0.12% AG, 12% GA, 0.3 % MC and 0.4% MC solutions show non-Newtonian behaviour whereas PBS, 10% GA and 0.2% MC indicates the Newtonian behaviour. The viscosity ranges were selected such that transition of fluid behaviour from Newtonian to non-Newtonian can be noted (refer Table 1).

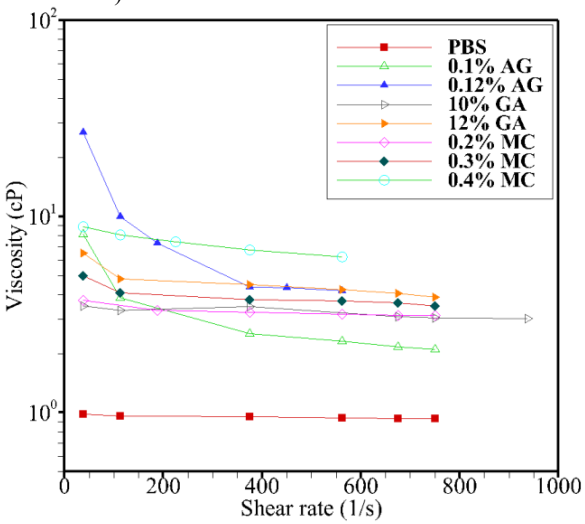

Figure 3: Relation between shear rate (1/s) and viscosity (cP). Newtonian fluids show constant viscosity with change in shear rate while non-Newtonian fluids show a change in viscosity with change in shear rate.

Table 1: Viscosity of solutions (at 37°C) and their behaviour at a shear rate of 375/s (N: Newtonian, NN: non-Newtonian), average VCL and VSL, and motility (RP-rapid progressive, SP-slow progressive, NP-immotile and non-progressive).

Fluid	Viscosity (cP)|Behaviour	Average VCL|VSL (μm/s)	% RP|SP|NP
PBS	0.74|N	171.0|43.8	55|18|27
0.1% AG	2.52|NN	329.2|58.5	68|21|11
0.12% AG	4.35|NN	318.2|56.8	61|22|17
10% GA	3.47|N	229.3|30.1	56|25|19
12% GA	4.51|NN	217.8|23.5	53|27|20
0.2% MC	3.24|N	230.0|65.2	56|28|16
0.3% MC	3.77|NN	278.0|67.2	75|13|12
0.4% MC	6.77|NN	184.9|45.3	56|26|18

4. RESULTS AND DISCUSSION

In this section, the effect of viscosity on the motion of cells is studied by quantifying them in terms of VCL and VSL. Moreover, based on the type of fluid, the motility parameters of the cells are classified in terms of rapid progressive (RP), slow progressive (SP), immotile and non-progressive (NP).

4.1 Effect of viscosity and behaviour of fluid on motion and motility of cells

Several experiments were carried out within a microchannel to investigate the role of fluid viscosity and fluid behaviour on the motion characteristics of the sperm cells. The quantified results in terms of individual velocity, average velocity and motility of sperm cells are shown in Fig. 4, Fig. 5 and Fig. 6.

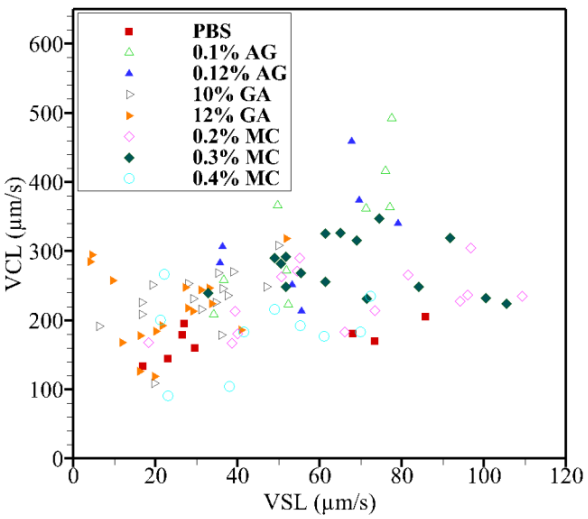

Figure 4: Curvilinear velocity (VCL) versus straight-line velocity (VSL). The scattered data points show cells in 0.3% MC have high VSL compared to other solutions.

Fig. 4 shows the individual values of VCL and VSL of sperm cells in various viscous solutions. The velocity behaviour of the sperm cells is strongly influenced by the surrounding fluid which can be interpreted from the distributed data points. The line passing through the origin and having a slope of 1.0 represents the ideal condition for sperm moving in a straight line. The data points of sperm velocities in PBS, 0.2% MC, 0.3% MC and 0.4% MC are distributed near to this line. This shows that the trajectory of the sperm in these fluids is close to the straight line. These scattered data points of sperm velocities may not give a clear and definitive understanding in different viscous environments. Hence, average velocities of sperm in each fluid are reported in Fig. 5.

The first set of experiments was conducted with PBS and the average velocity (VCL and VSL) values measured were 171 µm/s and 43.8 µm/s respectively. Next, the experiments were performed by adding 0.1% AG and 0.12% AG in PBS which showed non-Newtonian behaviour. The average values of VCL and VSL obtained with 0.1% AG were 329.2 µm/s and 58.5 µm/s respectively. In 0.12% AG, the average VCL decreased to 318.2 µm/s while VSL almost remained constant i.e., 56.8 µm/s. It is observed that, in the non-Newtonian fluids (0.1% AG and 0.12% AG), VCL decreases with an increase in viscosity. This decrease in velocity is a result of a higher drag force acting on the sperm at high viscosity. Next, experiments were conducted by using 10% GA and 12% GA in PBS. The behaviour of 10% of GA was Newtonian, but the behaviour of the 12% GA was non-Newtonian. For 10% GA, the average VCL and VSL obtained was 229.3 µm/s and 30.1 µm/s respectively. The VCL and VSL decreased to 217.8 µm/s and 23.5 µm/s respectively for 12% GA.

Next, experiments were performed with 0.2% MC, 0.3% MC and 0.4% MC. 0.2% MC showed Newtonian behaviour while 0.3% MC and 0.4% MC showed non-Newtonian behaviour. The obtained average values of VCL and VSL at 0.2% MC were 230 µm/s and 65.2 µm/s respectively. With an increase in viscosity, the average VCL increased to 278 µm/s while VSL almost remained constant i.e., 67.2 µm/s. Further, an increase in the viscosity resulted in decrease in average VCL and VSL to 184.9 µm/s and 45.3 µm/s respectively in 0.4% MC. The increase in VCL at 0.3% MC may be attributed to a change in the behaviour of fluid from Newtonian to non-Newtonian. Similar observations had been previously reported [13,14]. As cervical mucus is a non-Newtonian fluid, human sperm has evolved to migrate in a non-Newtonian fluid. While sperm is moving in shear thinning fluid, the sperm head creates a high shear rate that aligns randomly distributed polymer molecules along the direction of sperm motion which results in localized low viscosity around the tail thereby assisting sperm for rapid progression [23]. Due to this, sperm migration is more efficient in a non-Newtonian fluid. An exception for this behaviour is observed with 12% GA (non-Newtonian) which have lower velocities compared to 10% GA (Newtonian). This effect may be due to the presence of oxidase enzyme in GA. This enzyme promotes the reduction of oxygen in the fluid which may result in low sperm velocity and motility [22]. However, further study is required to explore the behaviour of sperm cells in GA.

From the above results, among all the viscous solutions, it was seen that 0.1% AG shows the highest average VCL of 329.2 µm/s and 0.3% MC shows the highest average VSL of 67.2 µm/s.

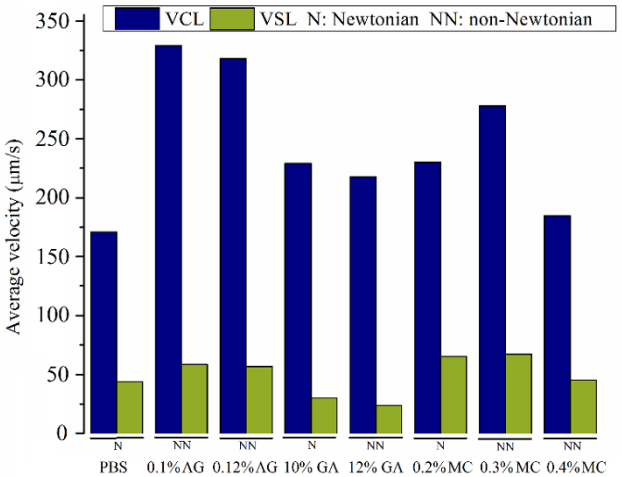

Figure 5: Average VCL and VSL in various viscous solutions. It shows that both velocities are increased by adding AG and MC in PBS while only VCL increased by adding GA in PBS.

In addition, along with velocity measurement, the values of sperm motility were calculated and classified in terms of their progression (%RP, %SP and %NP) as shown in Fig. 6. The values of RP, SP and NP obtained in PBS were 54.5%, 18.2% and 27.3%. After an increase in a solute concentration to 0.1% AG and 0.12% AG, the number of rapid progressive sperm cells increased compared to PBS as sperm have to exert more force to progress at high viscosity. It shows that 0.3% MC has the highest percentage of rapid progressive sperm cells (75%) followed by 0.1% AG which has 68%. It also shows that 10% GA and 12% GA has the lowest percentage of rapid progressive cells i.e., 56% and 53% respectively.

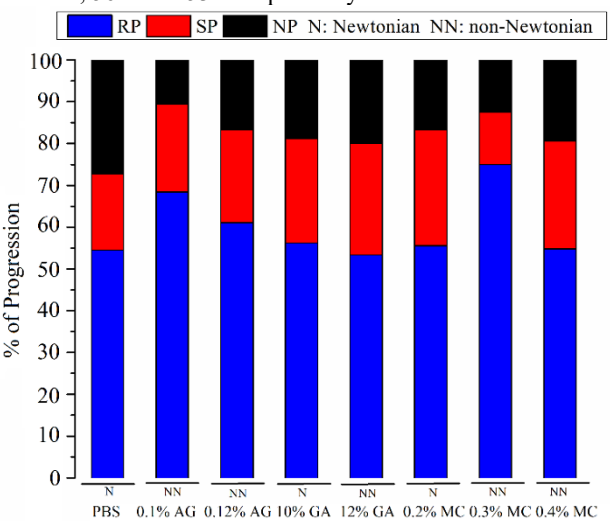

Figure 6: Motility of cells in different fluids. The motility of cells (rapid and slow progressive) increases by adding AG, GA and MC in PBS.

The quantified results shown in Fig. 5 and 6 indicate that the viscous environment of the surrounding fluid affects the

velocity and motility in a significant manner. Although PBS has a low viscosity value, the percentage of progression and VCL was least for sperm in PBS solution. Obtained results indicate that shear thinning fluids promote progression and motility of sperm. Further, it is also observed that sperm motility is higher in non-Newtonian fluids compared to Newtonian fluids except for 12% GA. In non-Newtonian fluids, sperm motility decreases with an increase in viscosity. The exceptional behaviour was observed in 12% GA due to the presence of oxidase enzyme which retards the flow of cells.

4.2 Motion characteristics in non-Newtonian fluids

The non-Newtonian fluids 0.12% AG, 12% GA and 0.3% MC were selected for the study of motion characteristics. Fig. 7 (a), (b) and (c) shows sperm cells swimming in a straight microchannel with their tails highlighted in blue colour in 0.12% AG, 12% GA and 0.3% MC respectively. To analyse the effective progression of sperm, determination of amplitude of the sperm trajectory and linearity are key factors. The high linearity of the sperm indicates the straight-line progression of sperm and high values of amplitude shows a stronger deviation of cell from their mean progress axis.

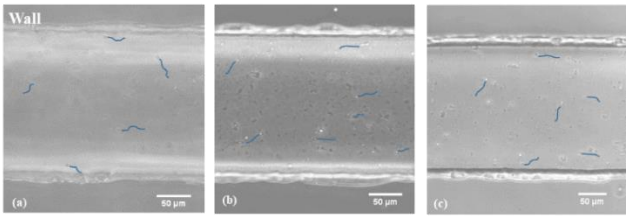

Figure 7: Sperm cells swimming in non-Newtonian fluids. (a) 0.12% AG (b) 12% GA and (c) 0.3% MC (Scale bar: 50 µm).

Fig. 8(a) shows the amplitude versus linearity relation of cells in 0.12% AG, 12% GA and 0.3% MC. Fig. 8 (b), (c) and (d) shows the relationship between linearity and amplitude of a sperm cell trajectory in 0.12% AG, 12% GA and 0.3% MC. Here, high average linearity is observed in fluid 0.3% MC i.e., 0.25 compared to that of 0.18 and 0.11 in 0.12% AG and 12% GA respectively. This indicates that in 0.3% MC, sperm cells migrate in a straighter path compared to 0.12% AG and 12% GA. The average amplitude of cell trajectory in 0.3% MC, 0.12% AG and 12% GA are 5.03, 4.6 and 4.26 µm respectively. Hence in the case of non-Newtonian fluids, an increase in amplitude and linearity leads to an increase in VSL which results in rapid progression of cells.

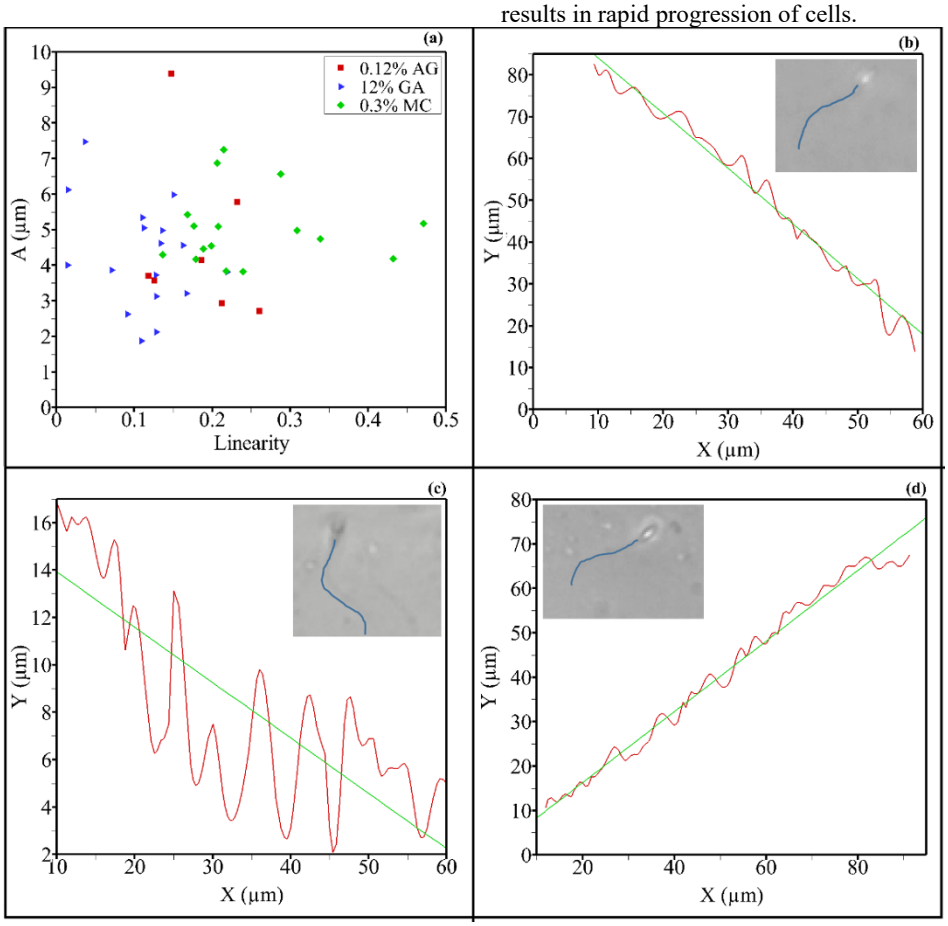

Figure 8: (a) Amplitude (A) versus Linearity of cells (b) Trajectory of a sperm cell in 0.12% AG (c) Trajectory of a sperm cell in 12% GA and (d) Trajectory of a sperm cell in 0.3% MC. In (b), (c) and (d), the image of a single sperm cell with tail highlighted in blue is shown. The red curve and the green line represent the sperm trajectory and the progress axis of the sperm obtained by using the least-squares method from the trajectory point.

5. CONCLUSIONS

The experimental observations and results show that the velocity and motility of sperm cells strongly depend on the viscosity and type of surrounding fluids. Among the fluid type considered, the sperm cells in non-Newtonian fluids have higher curvilinear velocity (VCL) and straight-line velocity (VSL) compared to Newtonian fluids with exception for 12% GA. In non-Newtonian fluids, an increase in viscosity leads to a decrease in VCL. Sperm cell motility is also found to be influenced by the surrounding fluid. 0.3% MC solution has the highest percentage of rapid progressive motility (75%) and 12% GA has the lowest percentage of rapid progressive motility (53%). The motion characteristics of sperm cells show that higher amplitude of sperm trajectory leads to increased linearity in non-Newtonian fluids. Sperm cells in 0.3% MC possess the highest average linearity and amplitude and therefore they show the highest percentage of rapid progression. The findings of this study will provide a better understanding of the effect of viscosity and type of surrounding fluid on sperm motion in a microchannel. In future, these fluids and the results obtained will be utilized for designing a microfluidic device for the separation and isolation of high-quality progressive sperm cells.

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ETHICAL CLEARANCE

All experiments were conducted following the rules and regulations approved by an ethical committee, Birla Institute of Technology and Science-Pilani, K K Birla Goa Campus.

NOMENCLATURE

γ	Shear rate	[1/s]
μ	Viscosity	[cP]

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