



RESEARCH ARTICLE

EFFECT OF GLUCOSE ON SPERM MOTILITY IN *DANIO RERIO* AS A MODEL OF DIABETES: A PRELIMINARY STUDY.

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Abstract

Diabetes mellitus impairs reproductive function by causing low sperm count, seminal volume, and sperm motility. Zebrafish (*Danio rerio*) is a potential model organism for diabetes, and the normal glucose levels in zebrafish and human semen are known. However, the glucose levels in semen that cause adverse effects in diabetes are unclear. Therefore, this study aimed to evaluate whether there is a direct effect of glucose on sperm motility and determine the glucose concentration that shows a significant effect on sperm motility and morphology by mimicking diabetes-like conditions in zebrafish *in vitro*. A two-part experiment was conducted: (a) Quantitative study, wherein sperm motility was examined at different glucose concentrations. (b) Qualitative study, wherein sperm morphology was visualized using Eosin Y staining. Data were analyzed using analysis of variance and post-hoc Dunnett's test. The results showed that sperm motility significantly diminished with 5-h incubation in 60 mM glucose. Concentrations of 1–5 mM glucose did not have any effect on sperm motility at 2, 3, or 5 h. However, several morphological abnormalities were observed across glucose concentrations and incubations. In conclusion, glucose at a concentration of 60 mM with 5-h incubation shows a change in zebrafish sperm motility. These findings may contribute to the development of zebrafish as a model of diabetes.

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Introduction:-

Currently, 347 million people are affected by diabetes worldwide, and the World Health Organization (WHO) predicts that diabetes will be the 7th leading cause of death in 2030. Diabetes mellitus has serious adverse effects on several organs of the body (World Health Organization, 2017), and studies focusing on the deleterious effects of diabetes on all body systems are required.

One of the main effects of diabetes is on the reproductive system. Diabetes leads to impaired reproductive function in men by disrupting important mechanisms at multiple levels such as spermatogenesis, steroidogenesis, sperm maturation, impairment of penile erection, and ejaculation, causing abnormal production of sperm or direct failure of reproductive function (Alves et al., 2013; Jangir and Jain, 2014). These impairments ultimately lead to a loss of motility, morphological integrity, impaired cell function, and apoptosis in sperm (Asmata et al., 2016; Bansal and Bilaspuri, 2010).

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Zebrafish is used as a model organism for several diseases, and research is now looking at developing it as a model for metabolic diseases like diabetes (Seth et al., 2013; Intine et al., 2013). Fish sperm, similar to human sperm, use fructose as the energy source after ejaculation. However, they can also use other sugars like glucose to a certain extent (Termer and Korsh, 2005). If glucose is added to the semen, the spermatozoa have a choice of glycolyzable substrates (Mann, 1946).

Under normal conditions, the concentration of glucose in males semen is 0.41 mM (Povoa et al., 1986) and that of fructose is 15 mM. In diabetes, the level of fructose can increase two- to four-fold, upto 30–60 mM and that of glucose can increase up to <1/30th the fructose levels, i.e., <2 mM (National Academies of Sciences, 1969). A previous study reported that in cyprinids, the normal level of glucose in semen is 1.094 mM (Aramli et al., 2013). Another study showed that the level of glucose in semen of grass carp (another cyprinid) is 0.07 mM (Bozkurt et al., 2008). Further, in certain species of teleosts fish, the concentrations of glucose are more than five times the concentrations of fructose (Piironen and Hyvärinen, 1983). These findings indicate that seminal concentrations of glucose vary across different species of fish.

Most studies on diabetes are *in vivo* observational studies, owing to ethical problems, and there is a need for a model organism in which *in vitro* studies on diabetes can be conducted. Therefore, this study aimed to evaluate the use of zebrafish as a model organism for diabetes in order to determine (a) whether there is an effect of glucose on sperm motility and (b) the concentration of glucose that shows a significant effect on sperm motility and morphology.

Materials and Methods:-

Zebrafish:-

Adult, wild-type *Danio rerio* (zebrafish) were used. The fish were purchased from a private aquarium and maintained in a 14 × 9 × 9 inch tank with a filter pump. The fish were fed flake food twice a day and maintained at ~28°C on a natural 12-h light:12-h dark photoperiod.

Sperm Maintenance:-

To maintain the testis containing sperm, sperm-immobilizing solution (SIS: 140 mmol/L NaCl, 10 mmol/L KCl, 2 mmol/L CaCl₂, 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), titrated to pH 8.5 with NaOH) was used (Kanuga et al., 2011). SIS mimics components of seminal fluid and keeps sperm immotile. Sperm were activated using sperm-activating solution (SAS: 40 mmol/L NaCl, 20 mmol/L HEPES, titrated to pH 8.5 with NaOH) (Kanuga et al., 2011). SAS mimics the inorganic components of ovarian fluid, which activates sperm and causes hypermotility to facilitate fusion of sperm and egg. Sperm were stored in SIS and activated with SAS.

Motility Estimation:-

The fish testes were dissected and stored in SIS on ice. Sperm motility was determined manually at 20 s after activation using phase-contrast microscopy (magnification, 10X; Olympus CX41, Olympus, India). The glass slides for microscopy were coated with 1.2% polyvinyl alcohol (Kanuga et al., 2011), and gaskets were placed over the slides for counting. To obtain images, imaging software Infinity Capture (Lumenera) was used. The total number of sperm visible in a field and the number of motile sperm were counted to determine % motility. Each sample was analyzed in triplicate.

Morphological Analysis:-

Sperm morphology was studied on a preliminary basis. Staining was performed using the Eosin Y staining method (World Health Organization, 2010). Briefly, sperm were mixed in a solution of 0.5% Eosin Y in 0.9% NaCl on a glass slide coated with 1.2% polyvinyl alcohol, following which the slide was covered with a 22 mm × 22 mm coverslip and left untouched for 30 s. The slide was examined with negative-phase-contrast optics (Olympus) at 40X magnification.

To obtain images, the same imaging software Infinity Capture (Lumenera) was used. The number of normal and abnormal sperm was tallied, and their averages were calculated and plotted graphically. Since this is a preliminary study, only 10 sperm were counted per sample. The Kruger criteria (Kandeel, 2007) are considered a universal standard by the WHO to differentiate between normal and abnormal sperms in humans, but no such criteria exist for zebrafish sperm. Therefore, we used the Kruger criteria to identify the sperm abnormalities in zebrafish sperm.

Glucose Concentration and Incubation Time:-

The effect of glucose was assessed by varying two main parameters: concentration of glucose and incubation period. Since the glucose levels in semen are far more relevant in diabetes, in this study, the glucose levels were focused upon. The effect of different concentrations of glucose (1–5 mM and 60 mM) with different incubation periods (1–5 h) was observed on sperm motility in zebrafish.

The levels of fructose in semen have been studied previously (Mann, 1946). As the fructose level in semen can increase up to 60 mM in humans, and the concentrations of glucose can increase upto more than five times the concentrations of fructose in some teleosts fish (Piironen and Hyvärinen, 1983), we used a maximum concentration of 60 mM glucose to account for this variation and determine how this extreme level of glucose would affect sperm motility. Sperm were first incubated with 60 mM glucose for 1-, 2-, 3-, 4-, and 5-h incubation periods. After observing statistically significant changes in motility at 5-h incubation and nearly significant changes at 2- and 3-h incubations, the glucose concentration was reduced to the normal level of glucose and increased gradually to a 5-fold higher concentration, i.e., 1, 2, 3, 4, and 5 mM, with 2-, 3-, and 5-h incubation periods, and the percent motility was calculated. For morphology studies, sperm were incubated with 1, 2, 3, 4, and 5 mM glucose for 2-, 3-, and 5-h incubation periods.

Statistical Analysis:-

One-way analysis of variance, with post-hoc Dunnett's test, was performed using Microsoft Office Excel (2007) to determine whether there were significant differences at various incubation times and concentrations of glucose. All p values < 0.05 were considered statistically significant.

Results:-**Motility studies:-**

Sperm incubated in 60 mM glucose for 5 h showed a remarkable reduction in motility compared to the control, whereas incubation for 2 and 3 h showed similar changes in motility (Fig 1). After statistical analysis, our results showed that incubation of sperm for 5 h with 60 mM glucose showed a significant change in motility ($p = 0.005$), and incubation periods of 2 and 3 h showed nearly significant changes as compared to the control, in that the value almost reached the critical significance level. However, concentrations of 1–5 mM glucose did not have any effect on the sperm motility at 2, 3, or 5 h (Fig 2).

Morphology Studies:-

The number of normal sperm incubated with different concentrations of glucose for different periods of time is presented in Table 1 and Fig 3. Selected images representative of each sample for each concentration and incubation period are presented in Fig 4–7. Owing to the small sample size and preliminary nature of the morphological study, no statistical analysis was performed on the quantitative morphological data.

Several types of abnormalities in sperm structure were observed. At 2-h incubation with 1–5 mM glucose, the most common abnormalities seen were short tails and no tails (Fig 4). A rare finding of a bifurcated tail was observed in the 2 mM glucose-incubated sample. At the 3-h incubation with 1–5 mM glucose, the most common sperm abnormalities seen were no tail, short tail, and terminal droplet. Notably, in some instances, a middle droplet was observed in 5 mM glucose-incubated sample and double tail was observed in the 5 mM glucose-incubated sample and 2 mM control sample. The number of normal sperm fluctuated widely among different concentrations (Fig 5). At the 5-h incubation with 1–5 mM glucose, terminal droplet, short tail, and no tail were the most common abnormalities observed. In the 1 mM and 4 mM control samples, bent tails were observed (Fig 6). At 1–5 h incubation with 60 mM glucose, the most common abnormalities were no tail, short tail, and terminal droplet. The rare abnormalities observed were joined tails and sperm heads of two sizes (Fig 7).

Discussion:-

In this study, the effect of glucose on sperm motility and morphology was examined. Our main finding from the motility study was that sperm incubated with 60 mM glucose for 5 h showed a significant reduction in motility. It may be possible that prolonged incubations of >5 h could also show an appreciable reduction in motility, giving glucose more time to interact with sperm. Furthermore, the lower incubation periods used in this study were probably insufficient to show any significant effect on sperm motility, as sperm are normally incubated in the fish testes for several weeks (Marshall, 2011). On further analyses, we found that none of the lower concentrations of

glucose, i.e., 1–5 mM, had an effect on sperm motility at 2, 3, and 5 h. The normal concentration of glucose in semen of cyprinids is 1.094 mM (Aramli et al., 2013), and concentrations of 1–5 mM were very close to the normal glucose values in fish semen. Thus, fish sperm may be able to tolerate these concentrations without showing a drastic effect on their motility. Alternatively, sperm might require longer exposure to glucose in order to show a significant change in motility, even with low concentrations of ~5 mM. Further studies should consider increasing the incubation time gradually to more accurately define the effects of glucose on sperm motility.

With regard to the morphological findings, we observed several sperm morphological abnormalities. A large number of tailless sperm were observed, which could be due the low magnification used, owing to which the tails may not have been clearly discernible. Another notable finding was the presence of sperm terminal droplets, i.e., excess residual cytoplasm from germ cells collected at the tail end. In humans, clinicians consider all cytoplasmic droplets as abnormal (Cooper, 2005). Cytoplasmic droplets are usually removed in spermiation during epididymal transport (Bostwick and Cheng, 2014). However, since in this study, we did not squeeze out the ejaculate from the fish, but instead dissected the testes to remove the sperm, this finding may not be related to the effect of glucose. Further studies should consider using the sperm ejaculate for experiments to verify this finding. A large proportion of shortened tails were also observed, but it is improbable that a genetic condition accounted for all of them. Thus, the cause of the large number of shortened tails in sperm in zebrafish still needs to be explored further.

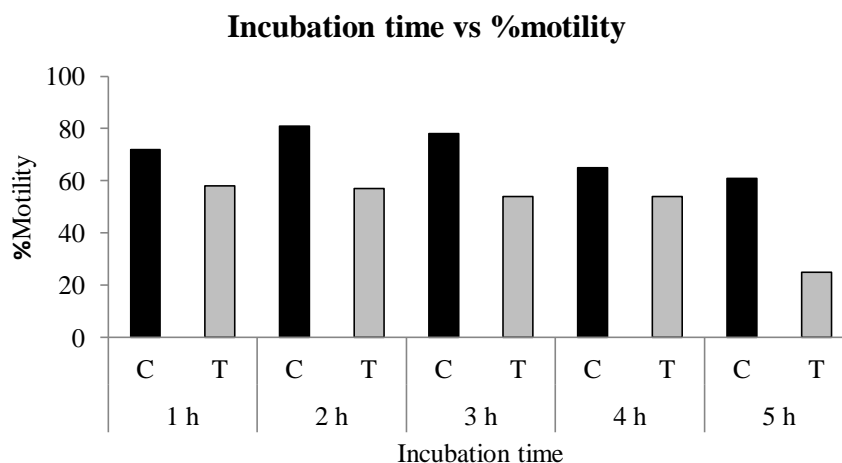
In conclusion, this study showed that a concentration of 60 mM glucose showed a significant reduction in sperm motility at 5-h incubation. Since this is a preliminary study, further work needs to be done to advance this research. The findings of this study may contribute to the development of zebrafish as a model for studying diabetes and help progress research on preventing the deleterious effects of diabetes on reproductive function in men.

Table 1:- Percentage of normal sperm from all experiments with different concentrations of glucose incubated for different periods of time (n = 10)

Time	Percent motile sperm									
	1 mM		2 mM		3 mM		4 mM		5 mM	
	C (%)	T (%)	C (%)	T (%)	C (%)	T (%)	C (%)	T (%)	C (%)	T (%)
2 h	90	100	100	90	80	90	90	100	90	100
3 h	60	90	80	70	60	100	30	50	60	50
5 h	50	70	70	70	60	60	50	70	60	60

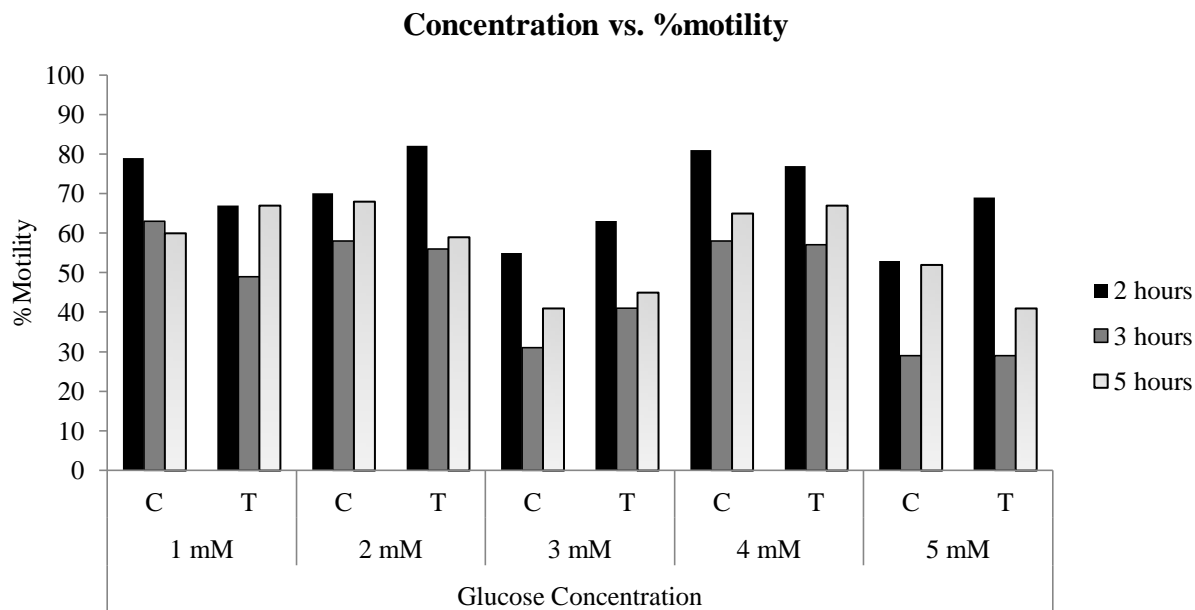
C: control; T: test (glucose)

Fig 1:- Graph showing the number of motile sperm on incubation with 60 mM glucose at 1–5 h



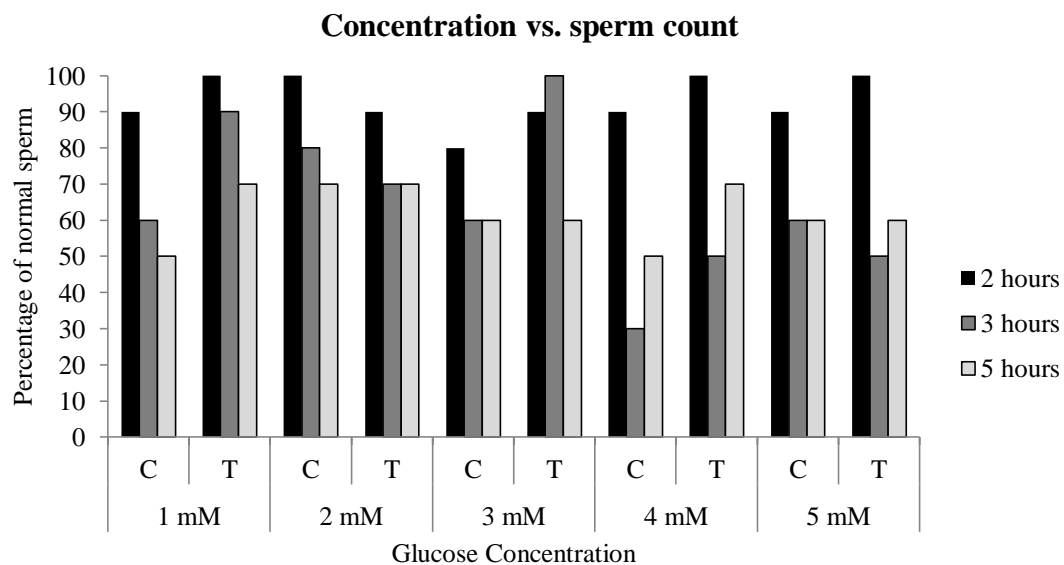
Black bars indicate the control. Grey bars indicate the test. C, control; T, test (glucose)

Fig 2:- Graph showing the number of motile sperm on incubation with 1–5 mM glucose at 2, 3, or 5 h



C, control; T, test (glucose)

Fig 3:- Graph showing the number of motile sperm on incubation with (A) 1–5 mM glucose at 2, 3, or 5 h and (B) 60 mM glucose at 1–5 h



C, control; T, test (glucose)

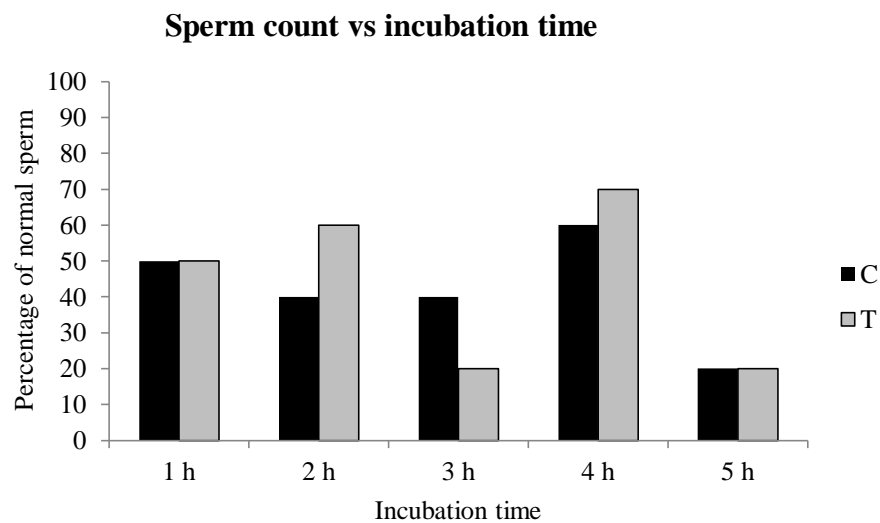


Fig 4:- Representative images of sperm stained with eosin Y at 2-h incubation with 1–5 mM glucose

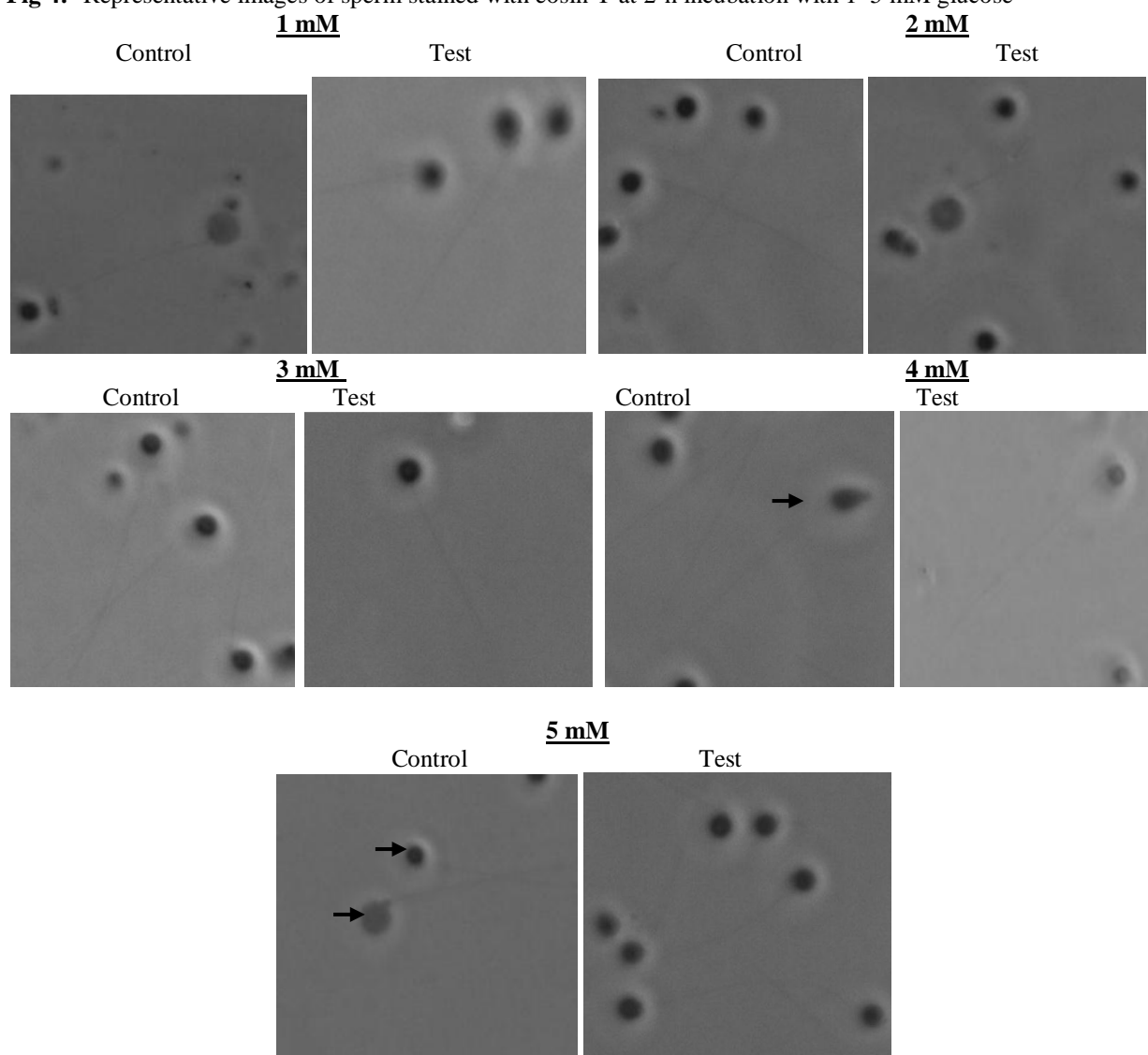


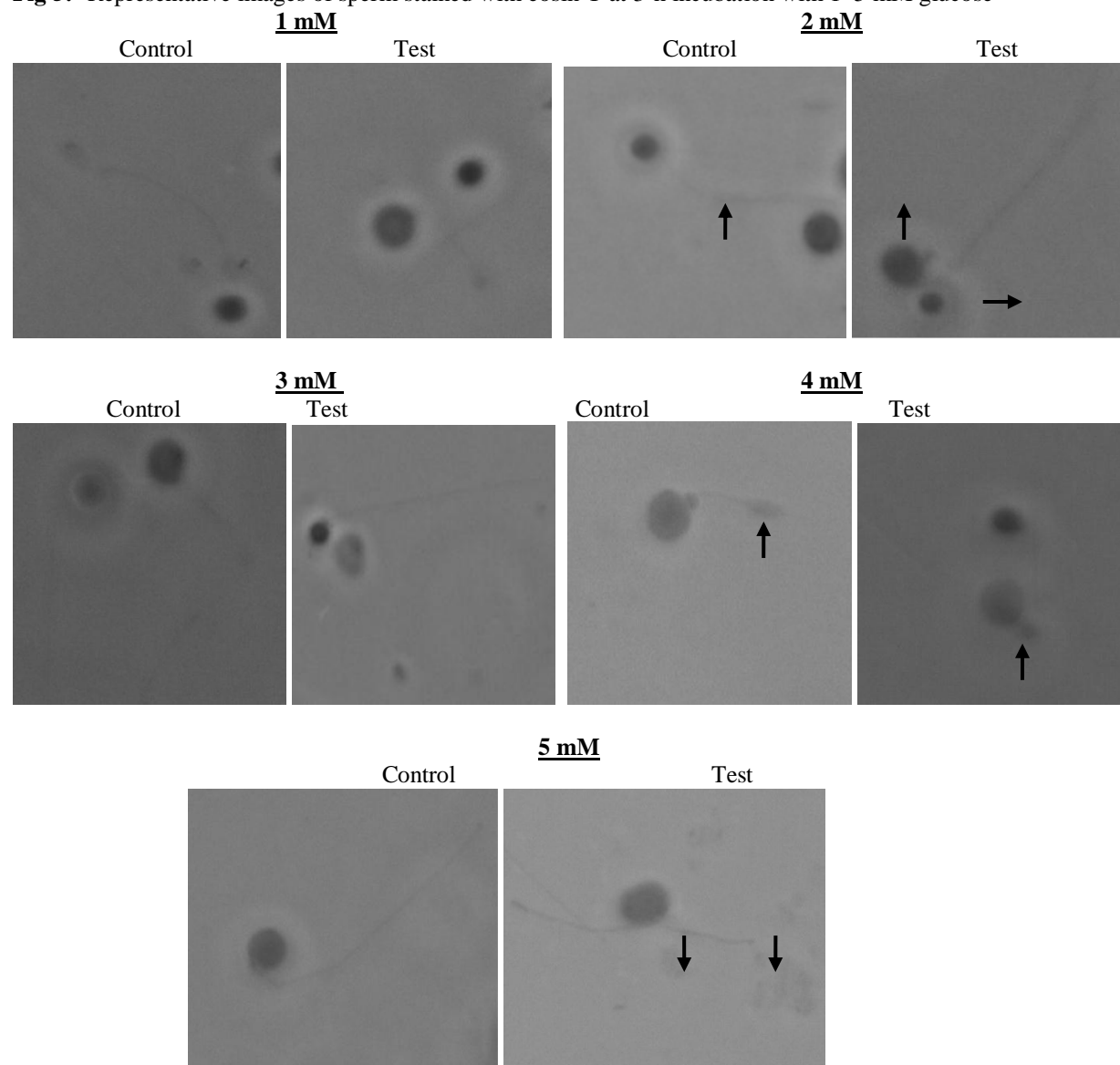
Fig 5:- Representative images of sperm stained with eosin Y at 3-h incubation with 1–5 mM glucose

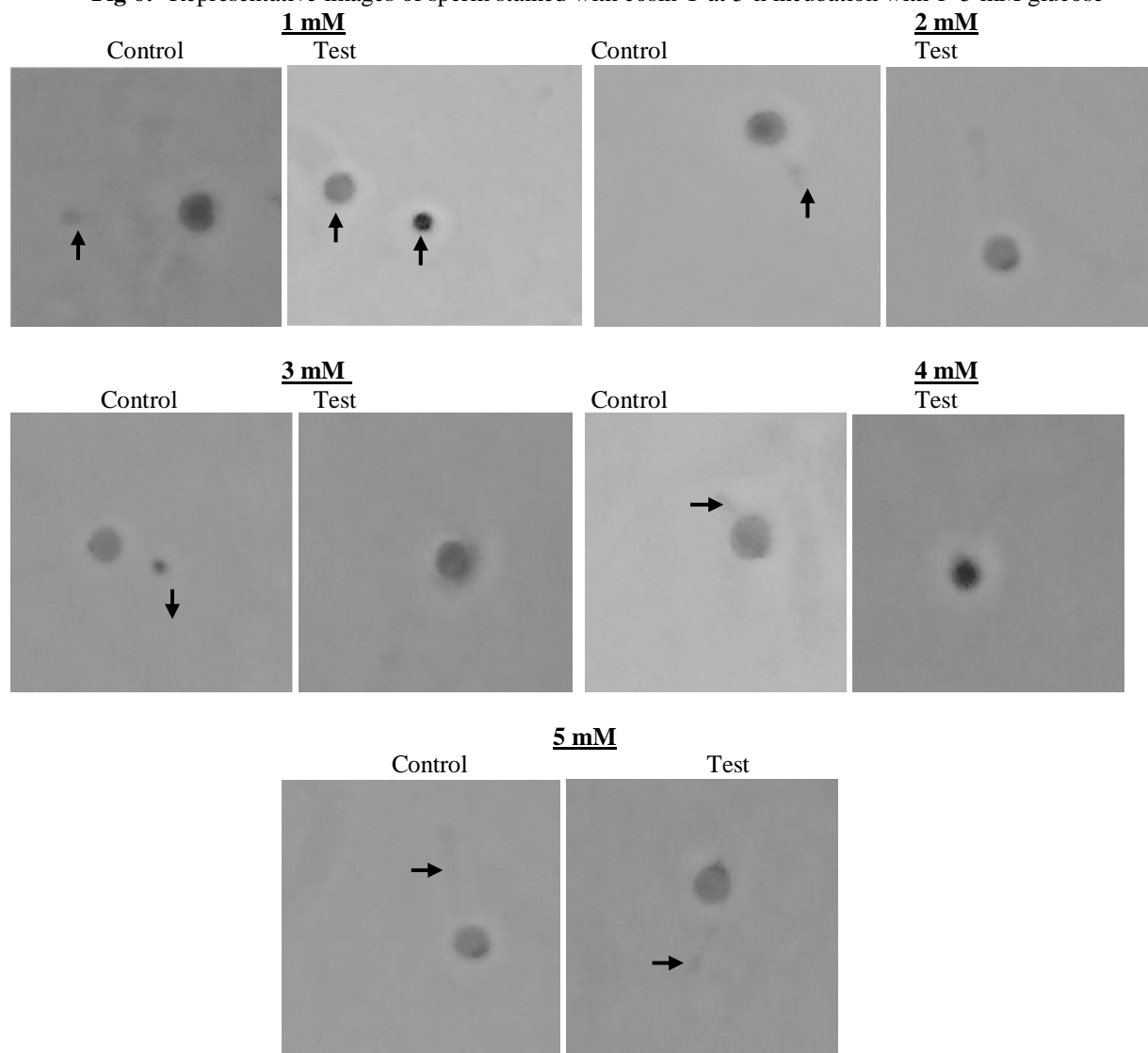
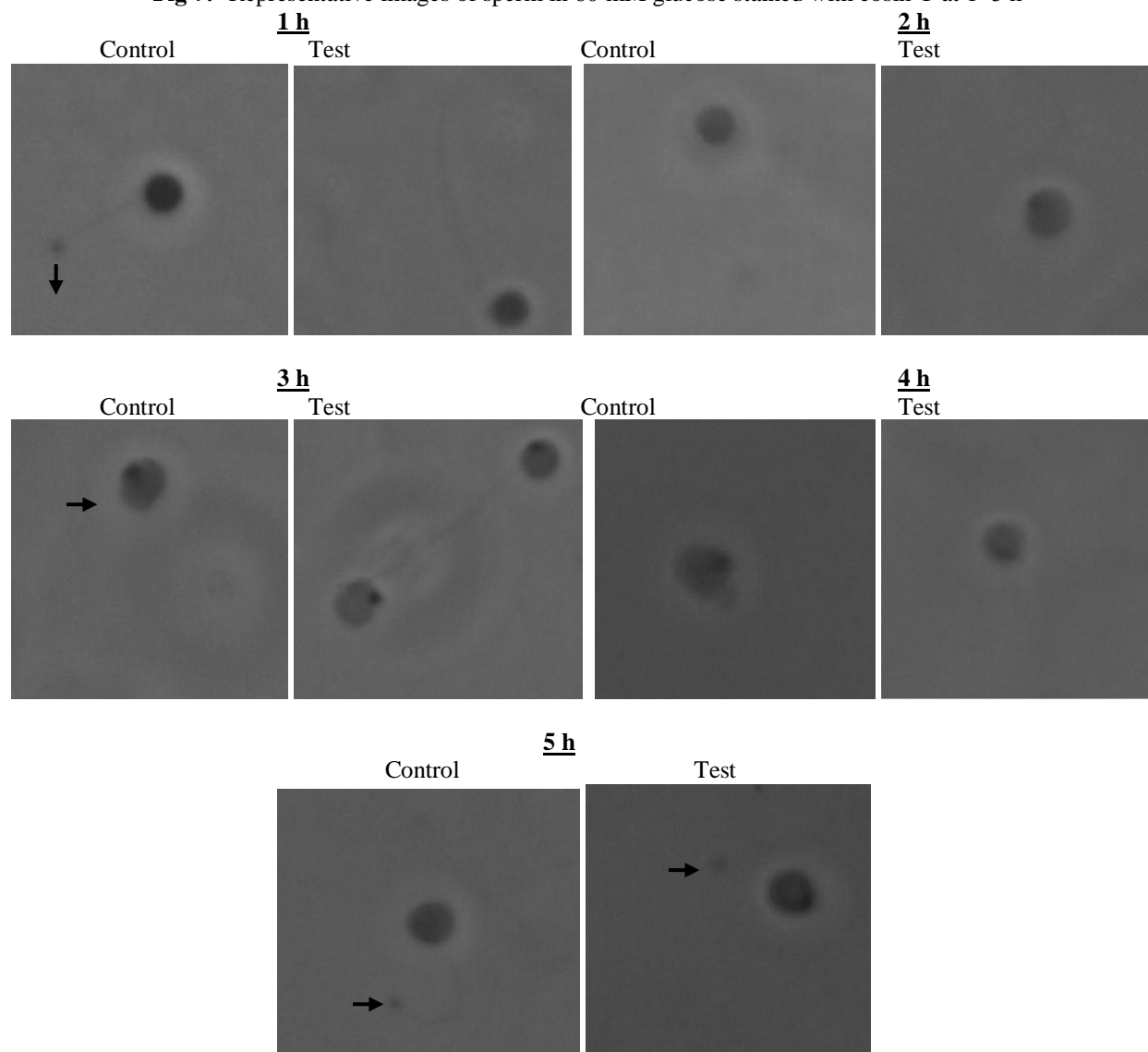
Fig 6:- Representative images of sperm stained with eosin Y at 5-h incubation with 1–5 mM glucose

Fig 7:- Representative images of sperm in 60 mM glucose stained with eosin Y at 1–5 h**Acknowledgements:-**

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