Effect of Different Metformin Doses on Post Thawing Human Sperm Motility and DNA Integrity

Wasan Adnan Al-jubory[1], Fatima Emad Al-nashme[2], Ramih Abdulfattah Al-Jobouri[3].

[1, 2, 3]Applied Embryology/ Higher Institute of Infertility Diagnosis and ART/ Al-Nahrain University/ Iraq.

E-mail: fatima_alnashme@yahoo.com

Abstract

Overview: Cryopreservation can cause permanent damage to sperm such as reduced motility, affect DNA integrity, acrosomal damage and plasma membrane deterioration. To improve spermatozoa function post thawing, several additives have been added into cryopreservation media and found to positively effect on viability and DNA integrity of frozen sperm. The current study was conducted for the inspection of using metformin as an additive to soothe the deleterious effect of cryodamage on sperm motility post thawing.

Method: Sixty-four semen sample from normozoospermic male used and all tested for DNA integrity. Each sample divided into three portions (control, 2.5 µM and 0.5 µM metformin). The control portion cryopreserved directly, while the other portions incubated for 30 minutes with metformin. Then, cryopreserved in LN2 for 48 hours. After thawing semen analysis and DNA integrity were done for each portion.

Results: There was a significant improvement in post thaw sperm parameters regarding motility and DNA integrity when used 0.5 µM compared to control group, while 2.5 µM showed no improvement compared with the control group.

Conclusion: Adding metformin (0.5 µM) as an additive to cryopreservation protocol improves semen parameters post thawing regarding motility and DNA integrity compared to the routine freezing protocol. While (2.5 µM) showed no effect on sperm.

Keywords: Cryopreservation, metformin, spermatozoa, motility, DNA integrity.

1. Introduction

Infertility is an increasingly global problem, couples that failed to achieve pregnancy following at least twelve months of ongoing, unprotected sexual activity are considered to undergoing infertility [1]. About 15-20% of couples at reproductive age are infertile and 50% of these cases are male caused infertility [2].

The physiological understanding of male fertility gives a structural framework that helps in treatment and diagnosis of male caused infertility. Factors that involve in male sub-fertility or infertility are found to be environmental, physiological and or genetic causes Adewoyin [3]. Usually, the first sign of male infertility can be assets by analysis of seminal fluid [4].

Sperm cryopreservation has been widely used in assisted reproductive technologies (ART) and solves many issues in male fertility preservation [5]. However, regardless of the progress made over the years, the post-thaw quality and function of spermatozoa is reduced when compared to fresh sperm [6].

Cryopreservation can cause permanent damage to sperm such as reduced motility, affect DNA integrity, acrosomal damage and plasma membrane deterioration. The reasons behind these negative effects are generally originated from cold shock, oxidative stress, intracellular ice formation and hypertonic damage [7].

To improve spermatozoa function post thawing, several additives have been added into cryopreservation media and found to positively effect on viability and DNA integrity of frozen sperm [8]. One of these molecules is the biguanide, metformin, which is an anti-hyperglycemic medication used to treat patient with type two diabetes [9], also it has a role impacting metabolism control and has the capability to reduce reactive oxygen species and activation of transcription factor Nrf2 which in turn lead to increase the expression of antioxidant genes [9].

While at the cytoplasmic effect, metformin has the ability to 1) reduce the activity of mitochondrial complex 1, which results in lower the level of ROS [10]. 2) Metformin also activate the AMP- activated protein kinase which considered as a key regulator of the energy balance inside the cells [11], and by the activation of AMPK the cells switch it state from an anabolic to catabolic state [12]. 3) Metformin have a non-genomic action and it could be a beneficial additive to treat the sperm which considered as transcriptionally dormant cell and also a molecule that could rapidly adjust the metabolism of spermatozoa to adapt it to the surrounding environment [13].

2. Materials and methods

A sixty-four normozoospermic semen samples were used. All fresh samples were tested for DNA integrity using acidic aniline blue stain (AABS) then divided into three portions separately. The first portion was cryopreserved routinely as a control to compare with the other results later. The other two portions were incubated with metformin for 30 minutes in 37°C before freezing (the

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seminal plasma were removed by centrifugation for 3 minutes at 3000 rpm, and replaced by 0.5 and 2.5 µM metformin solution). All portions cryopreserved in liquid nitrogen -196 for 48 hours then thawed. Post thawing examination was applied for all samples including (SFA and DNA integrity assessment by using acidic aniline blue stain). Comparison was made between each group to evaluate the differences occurred due to the addition of metformin.

3. Results

Comparison between fresh, control, and 0.5 µM metformin frozen samples

Sperm parameters regarding concentration and motility in fresh samples, control frozen samples and samples frozen with 0.5 µM metformin were compared between each other and illustrated in Table (3.1). The mean of sperm concentration was found to be significantly lower (p < 0.05) in 0.5 µM metformin frozen samples than that in control and fresh samples.

Table 3.1: Comparison in sperm parameters between fresh, control, and 0.5 µM metformin frozen samples

Sperm parameters	Sample			F - value	p - Value
	Fresh Mean ± SD	Control Mean ± SD	0.5 µM Mean ± SD		
Concentration (106/ml)	30.51 ± 10.0	30.51 ± 10.0	24.23 ± 8.2	9.449	0.001
Progressive motility (%)	46.18 ± 12.0	26.04 ± 8.1	43.8 ± 11.4	68.251	0.001
Non-Progressive motility (%)	19.12 ± 7.3	13.64 ± 8.6	13.17 ± 5.6	13.257	0.001
Immotile sperm (%)	34.68 ± 10.9	60.15 ± 12.2	43.0 ± 13.2	73.323	0.001

On the subject of sperm mean progressive motility, there was a significant (p < 0.05) increase in fresh sample than the means in control and 0.5 µM metformin frozen samples (Figure 3.1).

In addition, the mean of sperm non-progressive motility was also significantly (p < 0.05) higher in fresh sample than the means in control and 0.5 µM metformin frozen samples (Figure 3.1).

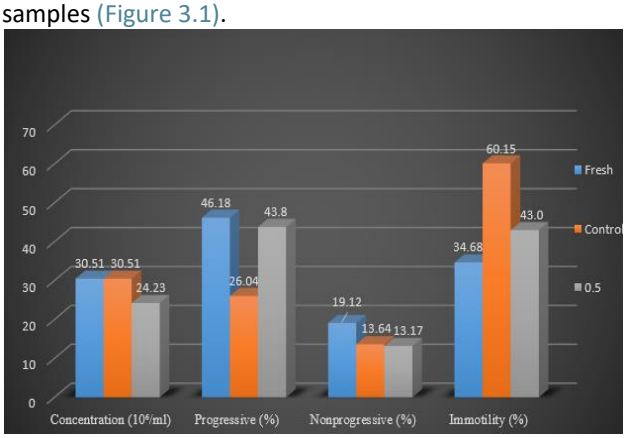

Figure 3.1: Sperm parameters compared between fresh, control, and 0.5 µM metformin frozen samples

While the mean of immotile sperm was found to be lower significantly (p < 0.05) in fresh samples than that in control and 0.5 µM metformin frozen samples (Figure 3.1).

Comparison between fresh, control, and 2.5 µM metformin frozen samples

The data collected from samples frozen with the addition of (2.5 µM metformin) to fresh semen samples were compared to data collected from fresh and control samples and a comparison was made to elucidate the effect and the results are shown in Table (3.2).

The mean of sperm concentration in 2.5 µM metformin frozen samples was significantly (p < 0.05) lower than that in control and fresh samples (Figure 3.2).

While, mean of sperm progressive motility of fresh samples was significantly higher (p-value, 0.001) than the parameters of control and 2.5 µM metformin frozen samples (Table 3.2).

Table 3.2: Comparison in sperm parameters between fresh, control, and 2.5 µM metformin frozen samples

Sperm parameters	Sample			F - value	p - Value
	Fresh Mean ± SD	Control Mean ± SD	2.5 µM Mean ± SD		
Concentration (106/ml)	30.51 ± 10.0	30.51 ± 10.0	24.21 ± 8.1	9.502	0.001
Progressive Motility (%)	46.18 ± 12.0	26.04 ± 8.1	25.46 ± 8.5	94.649	0.001
Non-Progressive motility (%)	19.12 ± 7.3	13.64 ± 8.6	16.82 ± 8.7	7.168	0.001
Immotile sperm (%)	34.68 ± 10.9	60.15 ± 12.2	57.6 ± 11.6	94.446	0.001

Moreover, the mean of the non-progressive sperm motility of fresh samples was significantly higher (p-value, 0.001) than that of both control and 2.5 µM metformin frozen samples (Figure 3.2).

Interestingly, control group showed a significant increase in the mean of immotile sperm percentage than both other groups (fresh and 2.5 µM metformin frozen samples), Figure (3.2).

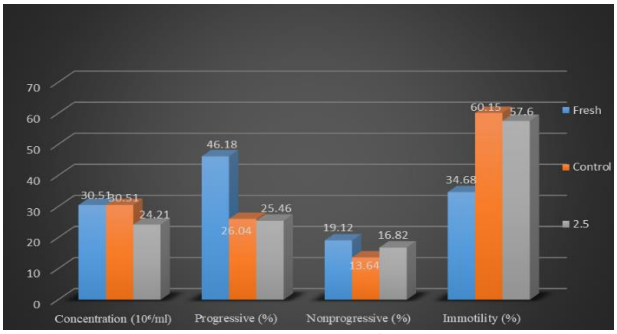

Figure 3.2: Sperm parameters in fresh, control, and 2.5 µM metformin frozen samples

Comparison between fresh, control, and (2.5 µM, and 0.5 µM) metformin frozen samples regarding DNA integrity

Sperm DNA integrity was compared between fresh semen samples, control frozen samples and frozen samples with 2.5 µM and 0.5 µM metformin (Table 3.3).

Table 3.3: Comparison in sperm DNA integrity between fresh, control, (2.5 µM, and 0.5 µM) metformin frozen samples

Sperm DNA integrity	Sample				F - value	p - Value
	Fresh Mean ± SD	Control Mean ± SD	2.5 µM Mean ± SD	0.5 µM Mean ± SD		

Normal (%)	75.2 ± 13.3	42.45 ± 13.2	38.6 ± 13.7	51.4 ± 13.8	94.728	0.001
Abnormal (%)	24.8 ± 13.3	58.0 ± 13.3	61.3 ± 13.8	48.6 ± 13.8	95.226	0.001

In term of the fresh samples, the mean of normal sperm DNA integrity was significantly ($p < 0.05$) higher than the DNA integrity of sperm in other samples. The mean of abnormal sperm DNA integrity was also significantly lower ($p < 0.05$) in fresh sample than that in other samples (Figure 3.3).

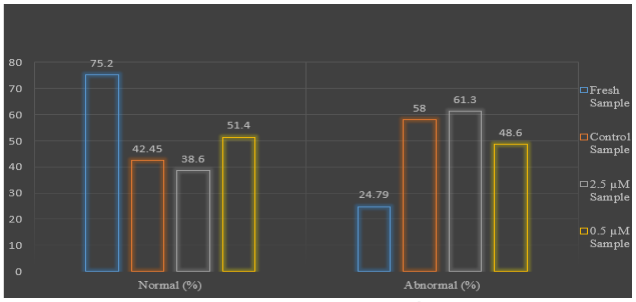

Figure 3.3: Sperm DNA integrity in fresh, control, (2.5 µM, and 0.5 µM) metformin frozen samples

4. Discussion

Characteristic of male patients in this study

All male patients included in this study fell into the category of normozoospermia as described by the WHO 2010. This criterion was chosen to reduce the variances in the samples compared to using oligozoospermic or asthenozoospermic samples, to ensure that the apparent effects on sperm parameters are due to only the use of metformin not to any other factor [14].

Effect of metformin on cryopreserved semen parameters

This is the first study that has evaluated the effect of metformin on cryopreserved human spermatozoa.

Table (3.1) and (3.2) showed that sperm concentration was not effected in fresh samples and in the control group when compared to the samples frozen with metformin, in which a significant decrease in sperm concentration was observed.

This decline in number of spermatozoa probably due to the technique used to prepare the sperm prior to freezing in which a low centrifugation force used to prevent undesirable interfering with the results regarding DNA fragmentation, which also was documented previously by other studies [15-17]. Regarding motility, there was no difference in adding metformin in 2.5µM when compared to the control group which indicated that the higher concentrations of metformin have no protective effect on sperm motility during cryopreservation.

A study was done by Londoño-Vásquez et al. [18]. on mice and bovine spermatozoa observed that the addition of high concentration of metformin 5000 µM showed no improvement in sperm motility after thawing [18]. While another study conducted on mouse noticed a slight reduction in sperm motility after thawing when using high metformin concentration (5000 µM). Similar results observed by Hurtado de Llera et al. when using (5Mm) metformin on boar sperm which also reduced motility [19].

On the contrary, a study conducted on chicken spermatozoa used a high concentration of metformin (1Mm) and observed a noticeable increase in the number of motile spermatozoa [20]. This in turn may indicate a species specific effect of metformin.

In the present study, a noticeable and interesting increase in sperm motility was observed when a low concentration used (0.5 µM) compared with the control.

Numerous studies have explained the damage that occur to cell organelles, such as mitochondria during the freezing and thawing procedure and this damage indicated due to the reduction in high membrane potential [21, 22]. Since the Restoration of sperm energy require intact mitochondria to provide the essential physiological ATP for sperm function post thawing, the current study hypothesized that the higher motility resulted indicate that metformin exerted a protective effect on mitochondrial activity against cryo injury, and this probably via the inhibition of mitochondrial complex 1 which also indirectly modulate AMPK activity.

Similar studies done on mice and canine found the same results, where they noticed that the lower concentration of metformin improve the motility post thawing, where it was suggested that the control of energy production and motility related to the stimulation of AMPK which found to be located in the head and mid-piece of sperm [23, 24].

Effect of metformin on sperm DNA integrity post thawing

The results showed in Table (3.3) point out that metformin with 0.5 µM could preserve sperm DNA integrity (p value 0.001), when compared to 2.5 µM and control samples, which suggest that metformin at this concentration had significant protective effects on sperm DNA.

This effect probably due to the anti-oxidative nature of metformin in which previous studies found that the addition of antioxidants to semen could be a beneficial to help reducing the deleteriousness of ROS production [25]. Several studies agreed with the finding of this study where they suggest that adding metformin as an additive slow down the oxidative stress and DNA fragmentation which could eliminate the negative effects on sperm chromatin integrity, and suggest that the elevation in ROS level is mainly due to damaged mitochondria where metformin found to maintain mitochondrial membrane potential which leads to decrease ROS generation [26] .

Metformin has been found to exert an antioxidant power and this could be through two possible pathways. The first one, by maintaining the level of electron leakage through the aerobic metabolism at the mitochondrial level, especially at mitochondrial complex I. The leakage of electrons/proton through the mitochondrial electron transport chain that causes the partial reduction of (O2-) into (H2O2) instead of water, the increase in electron leakage has found to be related to mitochondrial dysfunction, which in turn increase ROS level [27]. The second pathway of metformin for lowering the ROS, is by decreasing the level of NAD+ which is the primary precursor for NADP+ that reduced to NADPH as one of the sources of ROS production [26, 28].

5. Conclusions

Due to the results appeared in the present study, the following conclusions were made:

Adding metformin (0.5 µM) as an additive to cryopreserved semen significantly improve sperm motility after thawing compared to the control group, while (2.5 µM) showed no effect on sperm.

Metformin in (0.5 µM) also protect sperm DNA from deleterious effect of freezing and thawing technique compared to the other groups.

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