Assisted reproductive technology (ART) including in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) has witnessed huge advancements in recent years to treat patients with infertility (Fernandes et al. 2015). Despite the advancements in ART treatments, the success rates are still sub-optimal and 1 in 7 couples still experiences infertility globally (Calhaz-Jorge et al. 2016). In ART procedures, maintenance and correct handling of oocytes are critical for successful embryo development and healthy live births. Existing research recognizes that postovulatory ageing and oxidative stress are the major contributors to the loss of oocyte competence in vitro (Marteil et al. 2009; Lord and Aitken 2013). Several lines of evidence suggested that oxidative stress and in vitro ageing can be linked to Ca²⁺ homeostasis impairment, apoptosis, mitochondrial dysfunction, increased failed fertilization, and poor embryo development (Martín-Romero et al. 2008; Takahashi et al. 2009; Tang et al. 2013). Ca²⁺ signals influence most of the principal events related to fertilization and embryo development in all investigated species (Miao and Williams 2012). Disruption of Ca²⁺ homeostasis by metabolic stress resulted from in vitro ageing has been linked to certain cellular events that ultimately lead to cell death (Clapham 2007). Therefore, any changes to Ca²⁺ dynamics in oocytes might lead to defective signalling events and eventually will lead to failed fertilization and abnormal embryo development (Fernandes et al. 2015). Recently, Fernandes et al. have examined the effects of in vitro stress on Ca²⁺ homeostasis in human oocytes, and they found that intracellular Ca²⁺ was increased in human oocytes when exposed to in vitro conditions for 2 h (Fernandes et al. 2015). To prevent this increase in intracellular Ca²⁺, drugs targeting the ATP sensitive potassium channels (K_{ATP}) were applied to oocytes to investigate their cytoprotecting effects. Glibenclamide, a K_{ATP} channel blocker, has been shown to reduce and prevent intracellular Ca²⁺ loading and thus provide cytoprotection in human oocytes (Fernandes et al. 2015). Protection by glibenclamide was suggested to be via inhibition of mitochondrial and plasmalemmal K_{ATP} channels (Fernandes et al. 2015). ATP-sensitive K⁺ (K_{ATP}) channels are K⁺-selective channels gated by intracellular ATP (Nichols 2006). They are suggested to regulate intracellular metabolic conditions and cellular membrane excitability (Nichols 2006). In many cell types, K_{ATP} channels are shown to regulate and resist metabolic stress (Fernandes et al. 2015). In oocytes, K_{ATP} channels have been recently identified and it has been suggested that inhibiting these channels in human oocytes might provide cellular protection against in vitro ageing (Du et al. 2010). As no studies have tested Ca²⁺ and plasmalemmal membrane potential dynamics in oocytes, it is important to study their dynamics to investigate the effects of in vitro ageing and oxidative stress on MII mouse oocytes. Also, this study aims to show intracellular Ca²⁺ and plasmalemmal membrane potential dynamics in cryopreserved mouse oocytes in vitro to see if they show similar trends to those in human oocytes. The effects of glibenclamide on intracellular Ca²⁺ and plasmalemmal membrane potential dynamics in cryopreserved mouse oocytes will also be investigated to see if glibenclamide has any protective effects on the tested mouse oocytes. Results of this study represent a single observation and they do not belong to a currently running project.

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A total number of 56 cryopreserved MII mouse oocyte were used as the subjects of this study. Cryopreserved oocytes were purchased from Embryotech, USA, and no experiments were performed on animals. Therefore, no ethical approval was required to work on the cryopreserved mouse oocytes. All experiments performed at the research laboratory of the Clinical Embryology Department at the Medical School, Ninewells, and took place from April 2018 to July 2018. On arrival, oocytes were frozen by utilizing a slow freeze method by the supplier (Embryotech, Haverhill, MA). According to the supplier, purchased oocytes were harvested from superovulated female B6C3F-1 MA). According to the supplier, purchased oocytes were slow freeze method by the supplier (Embryotech, Haverhill, MA). According to the supplier, purchased oocytes were harvested from superovulated female B6C3F-1 × B6D2F-1 mice 12 h post-hCG injection. Before each experiment, oocytes were thawed at the research laboratory of the Clinical Embryology Department at the Medical School, Ninewells, according to the supplier instructions. Briefly, straws containing 5–10 oocytes were removed from the cane and held for 1 min in a 37°C water bath and then removed and wiped dry. The contents of the straw were immediately expelled into a holding dish containing 1 ml of Quinn’s AdvantageTM Medium with HEPES (Orgio, Måløv (Malov), Denmark). Finally, after the warming process, oocytes in the holding dish were incubated in a non-gassed incubator at 37°C until use. Only metaphase II oocytes with normal morphology represented by the presence of zona pellucida, normal cytoplasm, and single polar body were included in this study. Oocytes with abnormal morphology and different maturation stages were excluded from the study subjects.

Oocytes were arbitrarily divided between experimental groups that were studied independently from each other in order to assess Ca2+ and plasmalemmal membrane potential levels:

(i) Negative control (in the absence of any compound)
(ii) Dimethyl sulfoxide DMSO 0.1% (used to dissolve glibenclamide and FCCP)
(iii) Glibenclamide (100 μM in 0.1% DMSO, a KATP channel blocker)
(iv) Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) 50 μM in 0.1% DMSO (oxidative phosphorylation inhibitor and chemical hypoxia inducer)
(v) FCCP 50 μM + glibenclamide 100 μM (this group was only included for oocytes stained with Fluo-3 AM)

Laser confocal microscopy imaging was used to image oocytes that were loaded with Fluo-3 AM Calcium indicator (0.5 μg/ml in Quinn’s AdvantageTM Medium with HEPES (Orgio), at 37°C for 60 min) and Di-8-ANEPPS plasmalemmal membrane potential probe (10 μM in Quinn’s AdvantageTM Medium with HEPES (Orgio), at 37°C for 10 min). After staining, oocytes were loaded into the wells of the ibidi slide (ibidi, Germany) containing 180 μl of Quinn’s AdvantageTM medium with HEPES (Orgio) without human serum albumin (to prevent oocyte from floating and moving during the analysis). No more than 5 oocytes were loaded into the slide well. After oocyte loading, the slide was transferred to the confocal laser microscope room by a transport incubator at 37°C. The ibidi slide containing the oocytes was placed on the confocal laser microscope stage inside an environmental chamber. The temperature of the microscope chamber was maintained at 37°C during all the preparation and analysis times. Each oocyte imaged using laser confocal microscopy coupled to an inverted microscope (Leica TCS SP5 II, Milton Keynes, UK) with a ×10 (numerical aperture 1.3) oil-immersion objective lens.

The intensity of fluorescence of whole oocytes on the equatorial plane was measured. The microscope was calibrated by the green calibration slide before each experiment. The intensity of fluorescence was described in arbitrary units (AU) covering a range from 0 to 60,000 AU. Ca2+ levels, plasmalemmal membrane potential, and cell morphology were imaged every 10 min for 2 h using an Argon/UV laser (excitation 480–505 nm/emission 520–610 nm). After the 2 h, the fluorescent intensity of each oocyte probed with Fluo-3 AM and Di-8-ANEPPS was measured using Leica X Life Science software. Intensity quantification was done by marking the first region of interest which is the oocyte (ROI 01) and duplicating that region elsewhere in the field to represent the measurement background (ROI 02). The intensity of the background (ROI 02) was then subtracted from ROI 01 value to give the true fluorescent intensity measurement in the oocyte. Numerical values in the excel sheet were taken for each oocyte over time by Leica X Life Science software (Leica Microsystems, Wetzlar, Germany). For Fluo-3 AM, an increase in the pink fluorescent intensity indicates higher intracellular Ca2+ (Mitochondrial and endoplasmic reticulum Ca2+). For Di-8-ANEPPS, an increase in the green fluorescent intensity indicates plasmalemmal membrane depolarization, whereas a decrease in fluorescent intensity suggests plasmalemmal membrane hyperpolarization. This process was repeated for each oocyte and time interval for all the tested groups. The parameters of image acquisition were similar for all examined oocytes. Unless otherwise specified, all reagents and chemicals used in this study were purchased from Sigma-Aldrich, St. Louis, MO.

For the data analysis, normality and assumptions were calculated by using SigmaPlot version 4, from Systat Software, Inc., San Jose, CA, to ensure the data were normally distributed. The Shapiro–Wilk statistical test was used for normality testing. Repeated-measures two-way analysis of variance (RM two-way ANOVA) was performed as the data had two variables (time and intensity). This was carried out using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, CA. Additionally, Tukey’s multiple-comparisons test was used to detect any statistical significance.
between the individual groups and the different time points within each group. A P value less than 0.05 was considered statistically significant.

A total number of 32 cryopreserved mouse oocytes were loaded with Fluo-3 AM dye to monitor the changes in intracellular Ca$^{2+}$ over time. A baseline measurement was taken for all the oocytes before the different treatments. After the baseline measurement, the oocytes were divided among different treatment groups.

In Fig. 1a, the untreated oocytes (negative controls) ($n=7$) demonstrated no increase in intracellular Ca$^{2+}$ over 120 min. The slight decrease in the fluorescent intensity (34.3 ± 6 AU to 21.6 ± 5.4 AU) over time in the untreated group was statistically insignificant compared to the baseline measurement ($P$-value = 0.784); see Fig. 1a. Also, there was no difference between negative control and oocytes treated with glibenclamide ($n=10$) ($P$ value = 0.996) (Fig. 1a). This indicates that glibenclamide has no effect on Ca$^{2+}$ in cryopreserved mouse oocytes; see Fig. 1a. FCCP ($n=6$) and FCCP + glibenclamide ($n=4$) groups showed an increase in Fluo-3 AM intensity and that increase was statistically significant when compared to the untreated oocytes with $P$ values of 0.0004 and 0.0001 respectively (Fig. 1a). No statistical difference was recorded between DMSO group ($n=5$) and untreated oocytes ($P$ value = 0.679) (Fig. 1a). Figure 1b represents images of confocal laser microscopy for the oocytes in the untreated group from depicted time points (magnification ×10). The variation in sample size among the groups was not intended for a specific reason but depended on oocyte availability at the experiment time. Our results are not in agreement with Fernandes et al. findings (Fernandes et al. 2015). In Fernandes et al. study, they showed that human oocyte experience intracellular Ca$^{2+}$ overloading in vitro (Fernandes et al. 2015). In Swiss CD1 mice, Haverfield J. et al. have shown that oocytes obtained from young and old mice show similar Ca$^{2+}$ levels in stores such as ER and mitochondria (Haverfield et al. 2016). Therefore, mouse oocytes may express different Ca$^{2+}$ dynamics from

Figure 1. Ca$^{2+}$ changes over time in MII cryopreserved mouse oocytes. Fluo-3 AM means intensity changes over time (minutes) in cryopreserved mouse oocytes (a). Negative control $n=7$ oocytes, FCCP $n=6$ oocytes, FCCP + glibenclamide $n=4$ oocytes, glibenclamide $n=10$ oocytes, and DMSO $n=5$ oocytes. RM two-way ANOVA and Tukey’s multiple-comparisons tests used to determine statistical significance between the individual groups and to compare each time point within the single group to the baseline point. *P-value > 0.05. Error bars represent SEM. Images of confocal laser microscopy for the oocytes in the untreated group from depicted time points (magnification ×10) (b).
those in human oocyte and are capable of sustaining Ca\(^{2+}\) stores in vitro. However, many studies including Haverfield et al. study show that in vitro stress and ageing have dramatic effects on Ca\(^{2+}\) oscillation patterns in mouse oocytes (Tosti 2006; Haverfield et al. 2016). We also demonstrate that glibenclamide in the concentration of 100 μM does not affect the intracellular Ca\(^{2+}\) trend in mouse oocytes. Our results are in agreement with Li et al. study, which showed that glibenclamide did not affect the resting Ca\(^{2+}\) of Raw 264.7 macrophages (Li et al. 2014). This observation could be explained as glibenclamide might specifically target mitochondrial K\(_{ATP}\) channels, not the plasmalemmal ones. Therefore, future research should consider both mitochondrial and plasmalemmal K\(_{ATP}\) channels with Ca\(^{2+}\) regulation in oocytes. FCCP and FCCP+glibenclamide groups showed a significant increase in intracellular Ca\(^{2+}\) and these findings are similar to those in Fernandes et al. and Buckler KJ et al. studies when they used oxidative phosphorylation inhibitors to induce increase in intracellular Ca\(^{2+}\) in human oocytes and rat carotid body type I cells (Buckler and Vaughan-Jones 1998; Fernandes et al. 2015). FCCP is an oxidative phosphorylation inhibitor capable of inducing severe metabolic stress and it has been shown by several studies that FCCP also induces an increase in intracellular Ca\(^{2+}\) (mitochondria and ER) in several cell lines including MII mouse oocytes (Buckler and Vaughan-Jones 1998).

To record plasmalemmal membrane potential changes, 24 cryopreserved oocytes were loaded with Di-8-Anneps dye. A baseline measurement was also taken for all the oocytes before the different treatments. After the baseline measurement, the oocytes were divided among different treatment groups.

Figure 2a illustrates a spontaneous decrease in fluorescent intensity over 120 min in the negative control (n = 4) (23.3 ± 0.7 AU to 9.1 ± 0.4 AU) with a P-value of <0.0001. That decrease in intensity indicates that plasmalemmal...
membranes of MII mouse oocytes experience significant hyperpolarization (more negative membrane potential). The glibenclamide group (n = 10) also showed a significant decrease in fluorescent intensity over 120 min and when compared to the untreated group no significant difference was detected (P value = 0.782); see Fig. 2a. DMSO group (n = 5) showed similar intensity trends compared to the untreated oocytes and the glibenclamide group (Fig. 2a). Moreover, the FCCP group (n = 5) showed increased Di-8-Annep dye intensity and that increase was statistically significant when compared with the untreated group (P-value = 0.002); see Fig. 2a. Figure 2b represents images of confocal laser microscopy for the oocytes in the untreated group from depicted time points (magnification×10). The variation in sample size among the groups was not intended for a specific reason but depended on oocyte availability at the experiment time. As data showed a significant decline in plasmalemmal membrane potential (therefore hyperpolarized) in all the groups, except for the positive control (FCCP) group. It has been suggested that FCCP activates ionic currents of H+ and Na+ in the cell and therefore depolarizes the plasma membrane potential in a dose-dependent manner (Park et al. 2002). This observation could not be explained concerning oocytes as to the best of our knowledge no previous published studies investigated plasmalemmal membrane potential in oocytes. Glibenclamide did not cause any changes in the hyperpolarized plasmalemmal membrane potential compared to the control groups. This observation suggests that glibenclamide might function exclusively on mitochondrial KATP channels, as suggested by Fernandes et al. study (Fernandes et al. 2015). To confirm this, higher concentrations of glibenclamide and longer incubation time are needed.

In conclusion, our data show no spontaneous Ca2+ increase in untreated cryopreserved mouse oocytes loaded with Fluo-3 AM dye in vitro. We also suggest that glibenclamide has no effects on Ca2+ homeostasis and plasmalemmal membrane potential in cryopreserved mouse oocytes. Finally, we show that Ca2+ dynamics in B6C3F1 mouse oocytes are not similar to those in human oocytes.

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