Assessment of Intracellular Calcium and Plasmalemmal Membrane Potential in Cryopreserved Metaphase II Mouse Oocytes

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Research note

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Abstract

Objectives: Ca^{2+} is critical for normal oocyte activation and fertilization, and any alteration to the Ca^{2+} homeostasis may lead to failed fertilization or even cell death. It has been shown that intracellular Ca^{2+} is increased in bovine and human oocytes when cultured in vitro. Additionally, ATP sensitive potassium channels have been characterised recently in human and Xenopus oocytes. Glibenclamide a K_{ATP} channel blocker was shown to protect human oocytes from Ca^{+2} overloading via inhibition of plasmalemmal K_{ATP} channels. This research note aims to demonstrate the effects of oxidative stress and in vitro ageing on the intracellular Ca^{+2} and plasmalemmal membrane potential dynamics in cryopreserved metaphase II (MII) mouse oocytes. Also, this study aims to show if glibenclamide (a K_{ATP} channel blocker) has a role in regulating intracellular Ca^{+2} and plasmalemmal membrane potential through K_{ATP} channels in cryopreserved metaphase II mouse oocytes.

Results: our data did not show an increase in intracellular Ca^{2+} in untreated cryopreserved mouse oocytes loaded with Fluo-3 AM dye. However, an increase in the plasmalemmal membrane potential was noticed (hyperpolarization). Glibenclamide has shown no significant effect on Ca^{2+} and plasmalemmal membrane potential.

Introduction

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 (1). Despite the advancements in ART treatments, the success rates are still sub-optimal and I in 7 couples still experience infertility globally (1). In ART procedures, maintenance and correct handling of oocytes are critical for successful embryo development and healthy live births Existing research recognises that postovulatory ageing and oxidative stress are the major contributors to the loss of oocyte competence in vitro (2,3). Several lines of evidence suggested that oxidative stress and in vitro ageing can be linked to Ca^{2+} homeostasis impairment, apoptosis, mitochondrial dysfunction, increased failed fertilization, and poor embryo development (4–6). Ca^{2+} signals influence most of the principal events related to fertilization and embryo development in all investigated species (7). Disruption of Ca^{2+} homeostasis by metabolic stress resulted from in vitro ageing has been linked to certain cellular events that ultimately lead to cell death (8). Therefore, any changes to Ca^{2+} dynamics in oocytes might lead to defective signalling events and eventually will lead to failed fertilisation and abnormal embryo development (1). Recently Fernandes et al. have examined the effects of in vitro stress on Ca^{2+} homeostasis in human oocytes, and they found that intracellular Ca^{2+} was increased in human oocytes when exposed to in vitro conditions for two hours (1). To prevent this increase in intracellular Ca^{2+}, 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 Ca^{2+} loading and thus provide cytoprotection in human oocytes (1). Protection by Glibenclamide was suggested to be

via inhibition of mitochondrial and plasmalemmal K_{ATP} channels (1). ATP-sensitive K+ (K_{ATP}) channels are K+-selective channels gated by intracellular ATP (9). They are suggested to regulate intracellular metabolic conditions and cellular membrane excitability (9). In many cell types, K_{ATP} channels shown to regulate and resist metabolic stress (1). 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 (10). As no studies have tested Ca^{2+} and plasmalemmal membrane potential dynamics in mouse 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 Ca^{2+} 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 Ca^{2+} 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.

Materials And Methods

MII mouse oocytes

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. At arrival, oocytes were frozen by utilizing a slow freeze method by the supplier (Embryotech, US). According to the supplier, purchased oocytes were harvested from superovulated female B6C3F-1 x B6D2F-1 mice 12 hours 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 minute in a 37^{o}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 HEPES buffered medium (Origio, Denmark). Finally, after the warming process, oocytes in the holding dish were incubated in a non-gassed incubator at 37^{o}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 Ca^{2+} 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 K_{ATP} 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

Oocytes were loaded with Fluo-3 AM Calcium indicator (0.5 µg/ml in HEPES medium, at 37˚C for 60 minutes) and Di-8-ANEPPS plasmalemmal membrane potential probe (10 µM in HEPES medium, at 37˚C for 10 minutes). Stains were freshly prepared on the day of the experiments. After staining, oocytes were loaded into the wells of the ibidi slide (ibidi, Germany) containing 180 µl of HEPES buffered media 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 60000 AU. Ca^{2+} 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 hours, 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, Germany). For Fluo-3 AM, an increase in the pink fluorescent intensity indicates higher intracellular Ca^{2+} (Mitochondrial and endoplasmic reticulum Ca^{2+}). For Di-8-ANEPPS, an increase in the green fluorescent intensity indicates plasmalemmal membrane depolarisation, whereas a decrease in fluorescent intensity suggests plasmalemmal membrane hyperpolarisation. 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.

Statistical Analysis

The normality and assumptions were calculated by using SigmaPlot version 4, from Systat Software, Inc., San Jose California USA 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 California USA. 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.

Results

Intracellular Ca^{+2} Changes in Cryopreserved MII Mouse Oocytes

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 Figure 1A the untreated oocytes (negative controls) (n=7) demonstrated no increase in intracellular Ca^{2+} over 120 minutes. 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 Figure 1A. Also, there was no difference between negative control and oocytes treated with glibenclamide (n= 10) (P value= 0.996) (Figure 1A). This indicates that glibenclamide has no effect on Ca^{2+} in cryopreserved mouse oocytes see Figure 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 (Figure 1A). No statistical difference was recorded between DMSO group (n= 5) and untreated oocytes (P value= 0.679) (Figure 1A). Figure 1B represents images of confocal laser microscopy for the oocytes of the different groups from depicted time points (magnification x 10). The variation in sample size amongst the groups was not intended for a specific reason but depended on oocyte availability at the experiment time.

Plasmalemmal Membrane Potential Changes in Cryopreserved Mouse Oocytes

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 minutes 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 hyperpolarisation

(more negative membrane potential). The glibenclamide group (n= 10) also showed a significant decrease in fluorescent intensity over 120 minutes and when compared to the untreated group no significant difference was detected (P value= 0.782) see Figure 2A. DMSO group (n= 5) showed similar intensity trends compared to the untreated oocytes and the glibenclamide group (figure 2A). Moreover, the FCCP group (n= 5) showed increased Di-8-Anneps intensity and that increase was statistically significant when compared with the untreated group (P-value= 0.002) see Figure 2A. Figure 2B represents images of confocal laser microscopy for the oocytes of the different groups from depicted time points (magnification x 10). The variation in sample size amongst the groups was not intended for a specific reason but depended on oocyte availability at the experiment time.

Discussion

In this study, we assessed the intracellular Ca^{2+} and plasmalemmal membrane potential trends over time in cryopreserved MII mouse oocytes to see if they show similar changes as those in human oocytes following data from Fernandes *et al.* study (1). Additionally, we investigated the glibenclamide effect on intracellular Ca^{2+}, and plasmalemmal membranes in cryopreserved MII mouse oocytes compared to controls.

This study shows that there is no spontaneous increase in intracellular Ca^{2+} in untreated cryopreserved mouse oocytes loaded with Fluo-3 AM dye. Our results are not in agreement with Fernandes *et al.*, findings (1). In Fernandes *et al.* study, they showed that human oocyte experience intracellular Ca^{2+} overloading in vitro (1). 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 (11). Therefore, mouse oocytes may express different Ca^{2+} dynamics than those in human oocyte and are capable of sustaining Ca^{2+} stores in vitro. However, many studies including Haverfield J. *et al* study show that in vitro stress and ageing have dramatic effects on Ca^{2+} oscillation patterns in mouse oocytes (11,12). 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 (13). 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 (1,14). 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 (14). Our data also demonstrates that plasmalemmal membrane potential significantly declined (therefore hyperpolarized) in all the groups, except for the positive control (FCCP) group. It has been

shown that FCCP activates ionic currents of H^+ and Na^+ in the cell and therefore depolarizes the plasma membrane potential in a dose-dependent manner (15). 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 K_{ATP} channels, as suggested by Fernandes et al. study (1). To confirm this, higher concentrations of glibenclamide and longer incubation time are needed.

Conclusions

Our data show no spontaneous Ca^{2+} increase in untreated cryopreserved mouse oocytes loaded with Fluo-3 AM dye in vitro. We also suggest that glibenclamide has no effects on Ca^{2+} homeostasis and plasmalemmal membrane potential in cryopreserved mouse oocytes. Finally, we show that Ca^{2+} dynamics in B6C3F1 mouse oocytes are not similar to those in human oocytes.

Limitations

- Unavailability of fresh mouse oocytes to compare with the cryopreserved ones.
- The low number of oocytes tested represents a limitation in the analysis and interpretation of the study findings.
- Ca^{2+} dynamics tested directly after thawing and staining of cryopreserved oocytes, therefore, longer incubation times before staining might give more insight into Ca^{2+} dynamics in MII cryopreserved oocytes.
- Only one concentration of glibenclamide was tested (100 µM in 0.1% DMSO) due to the low sample size.

Abbreviations

AU: arbitrary, Ca^{+2}: calcium, DMSO: Dimethyl sulfoxide, K_{ATP}: ATP sensitive potassium channels, MII: Metaphase II, mtDNA: mitochondrial DNA, HAS: Human Serum Albumin, HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) FCCP: carbonyl cyanide 4 (trifluoromethoxy)phenylhydrazone, DMSO: Dimethyl sulfoxide, SEM: stander error of the mean.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Conceptualization, OFA and TM; Methodology, OFA and TM; Investigation, OFA; Resources, TM; Writing–Original Draft, OFA; Writing–Review & Editing, OFA and TM; Visualization, OFA; Supervision, TM. All authors read and approved the final manuscript.

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2. Dr Therishnee Moodley is one of the modules lead on the Human Clinical Embryology and Assisted Conception MSc programme and an Honorary Senior Clinical Embryologist at Ninewells ACU.

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Figures

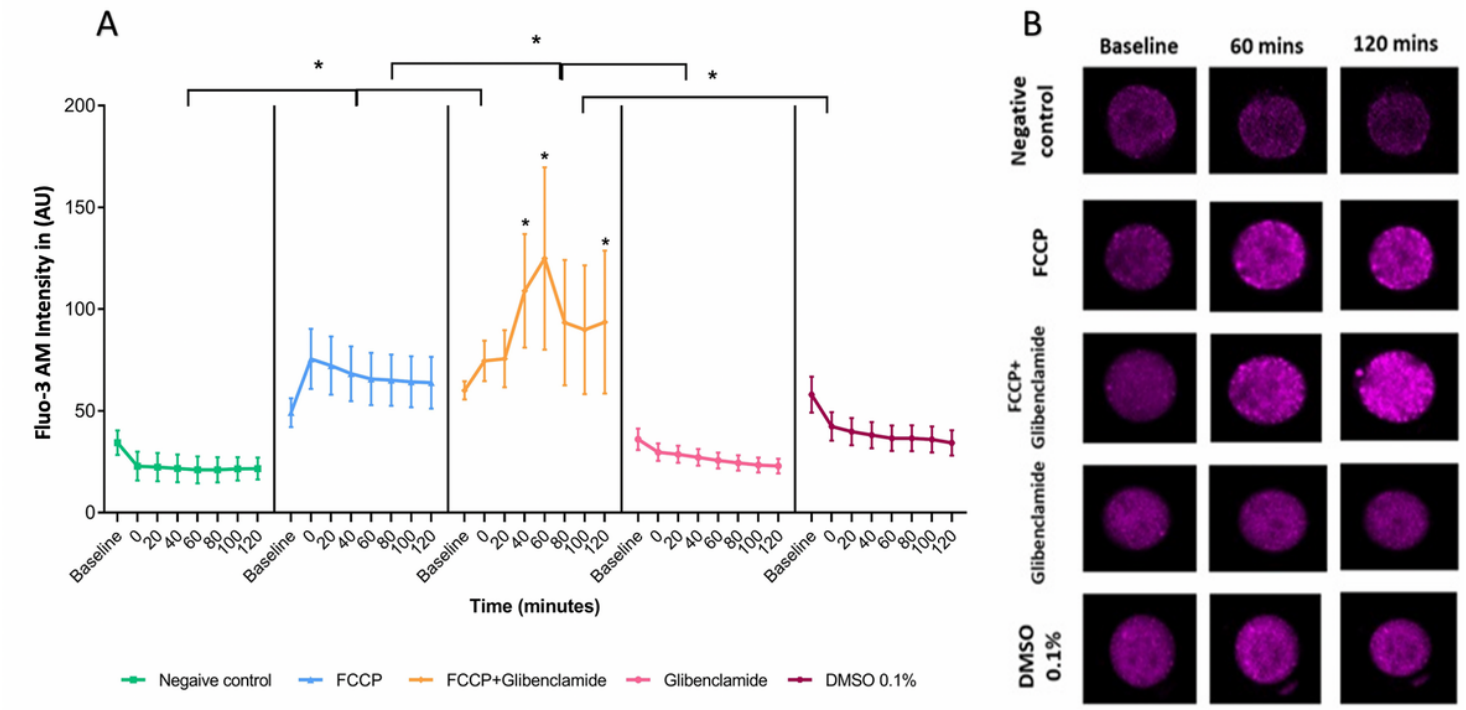

Figure 1

Ca2+ changes over time in MII cryopreserved mouse oocytes. (A) Fluo-3 AM means intensity changes over time (minutes) in cryopreserved mouse oocytes. 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 comparison 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. (B) Images of confocal laser microscopy for the oocytes of the different groups from depicted time points (magnification x 10).

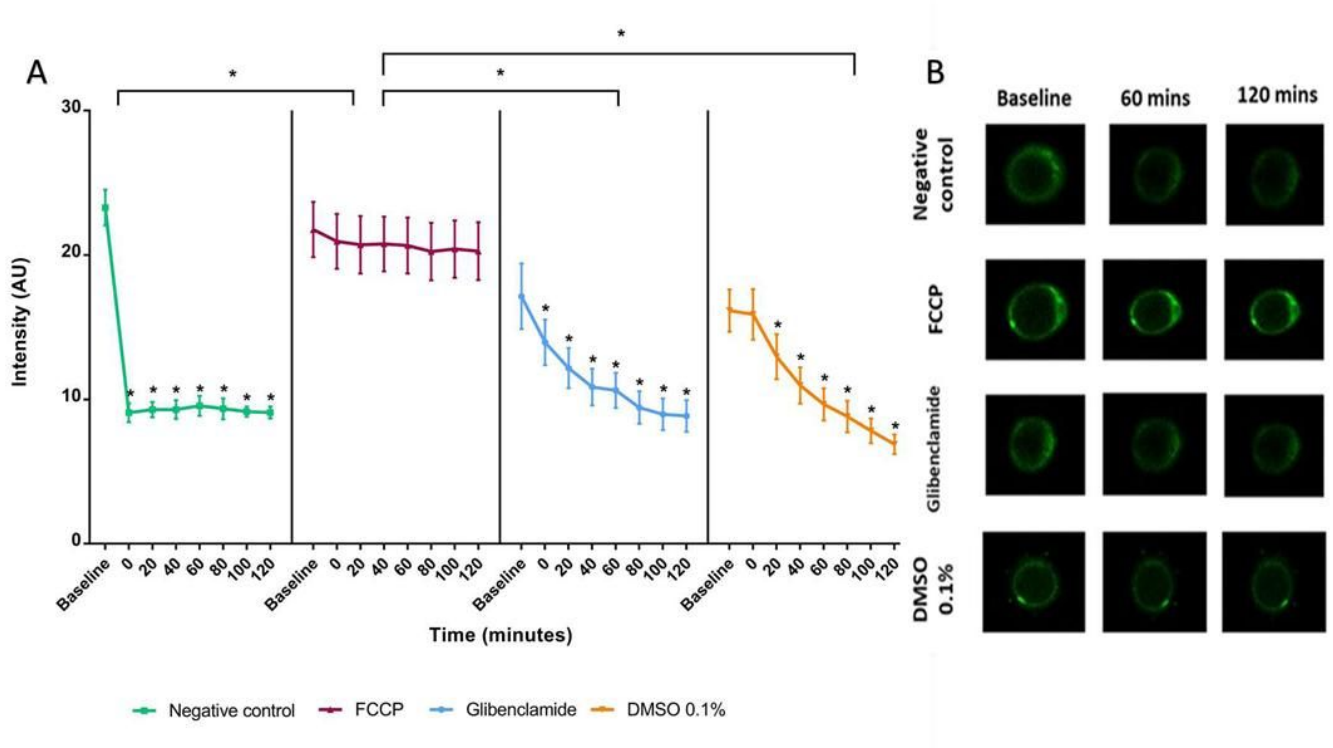

Figure 2

Di-8-Anneps changes over time in MII cryopreserved mouse oocytes. (A) Di-8-Anneps mean intensity changes over time (minutes) in cryopreserved mouse oocytes. Negative control n= 4 separate oocytes, FCCP n= 5 separate oocytes, Glibenclamide n=10 separate oocytes and DMSO n= 5 separate oocytes. RM Two-way ANOVA and Tukey's Multiple comparison tests used to determine statistical significant 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. (B) Images of confocal laser microscopy for the oocytes of the different groups from depicted time points (magnification x 10).

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