Reproduction, Fertility and Development

Metformin inhibits human spermatozoa motility and signaling pathways mediated by PKA and tyrosine phosphorylation without affecting mitochondrial function

Journal:	*Reproduction, Fertility and Development*
Manuscript ID	Draft
Manuscript Type:	Research paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Calle-Guisado, Violeta; Universidad de Extremadura, SINTREP/INBIO G+C González-Fernández, Lauro; Universidad de Extremadura, Biochemistry, Molecular Biology and Genetics Martín, David; University of Extremadura, SINTREP/INBIO G+C Garcia-Marin, Luis; University of Extremadura, SINTREP/INBIO G+C Bragado, Maria Julia; Universidad de Extremadura, SINTREP/INBIO G+C
Keyword:	spermatozoa, second messenger system, signal transduction, pharmacology

Metformin inhibits human spermatozoa motility and signaling pathways mediated by PKA and tyrosine phosphorylation without affecting mitochondrial function

V. Calle-Guisado, L. Gonzalez-Fernandez, D. Martin-Hidalgo, L. J Garcia-Marin*, M. J. Bragado*.

Research Group of Intracellular Signaling and Technology of Reproduction (SINTREP), Institute of Biotechnology in Agriculture and Livestock (INBIO G+C), University of Extremadura, 10003 Caceres, Spain.

* Both authors contributed equally as senior investigators of this study.

Running Title: Metformin inhibits human sperm motility

Corresponding author:

M. Julia Bragado, Ph. D

Research group of Intracellular Signaling and Technology of Reproduction (SINTREP)

Department of Biochemistry and Molecular Biology and Genetics, University of Extremadura, 10003 Caceres, Spain

ORCID: 0000-0001-7770-0775

Email: jbragado@unex.es

Abstract

Metformin is a leading anti-diabetic drug to treat the worldwide pathology of diabetes mellitus. This biguanide exerts metabolic and pleiotropic effects in somatic cell, although its in vitro actions in human spermatozoa remain unknown. This work investigates metformin effects on human spermatozoa function. Human spermatozoa were incubated in presence/absence of metformin for different times (8h-20h) and motility was evaluated by CASA whereas other functional parameters were evaluated by flow cytometry. Metformin significantly reduces the percentages of motile, progressive and rapid spermatozoa starting at 8h and significantly decreases sperm velocities. Metformin does not affect viability, mitochondrial membrane potential and mitochondrial superoxide anion generation in human spermatozoa. However, metformin clearly inhibits the PKA pathway and sperm protein tyrosine phosphorylation, key regulatory pathways for correct spermatozoa function. In summary, metformin treatment of human spermatozoa causes a detrimental effect in motility and inhibits essential sperm signalling pathways, PKA and protein tyrosine phosphorylation, without affecting physiological parameters (viability, mitochondrial membrane potential, mitochondrial superoxide anion generation). Given the growing clinical use of metformin in different pathologies besides diabetes, we think that this study might be very relevant in terms of human fertility in those patients that potentially could be treated with metformin in the future.

Key words: metformin, human spermatozoa, motility, mitochondria, PKA, tyrosine phosphorylation.

1. Introduction

Metformin is a compound isolated from *Galega officinalis*, the French lilac plant known to ameliorate the symptoms of diabetes mellitus. It has been used for more than 50 years and currently is a leading oral drug included in the biguanide class to treat diabetes mellitus type 2 (Coughlan *et al*., 2014; Hardie *et al*., 2013; Owen *et al*., 2000; Pernicova *et al*., 2014; Rosilio *et al*., 2014), a worldwide pathology that currently affects over 400 million adults, and is estimated to growth to over 600 million in 2040 (Cho, 2017; World Health Organization 2010). Metformin exerts several actions on insulin sensitivity in muscle and liver, including a decrease in hepatic glucose production, an increase in peripheral glucose utilization, and positive effects on insulin receptor expression and tyrosine kinase activity (Lee *et al*., 2012). Metformin also reduces insulin resistance by inducing translocation of the glucose transporter 4, GLUT4 (Lee *et al*., 2012). However, its beneficial effects may also include its anti-inflammatory (Martin-Montalvo *et al*., 2013) and anti-cancer actions (Hardie *et al*., 2014). Although metformin action was originally thought to be mediated by AMP-activating kinase, AMPK (Zhou *et al*., 2001), likely through inhibition of AMP desaminase (Ouyang *et al*., 2011), it is currently established that many of metformin effects are AMPK-independent (Ben-Sahra *et al*., 2011; Corominas-Faja *et al*., 2012; Kelly *et al*., 2015; Miller *et al*., 2010; Rosilio *et al*., 2014; Saeedi *et al*., 2008; Scotland *et al*., 2013). Thus, besides effects on glucose metabolism, metformin also directly inhibits complex I (NADH:ubiquinone oxidoreductase) of the mitochondrial electron transport chain (El-Mir *et al*., 2000; Owen *et al*., 2000) with obvious consequences in cell energy production. However, a debate highlighted the positions for (Hardie *et al*., 2006) and against (Hoek, 2006) the notion that metformin inhibits mitochondrial energy production. The real and potential benefits of metformin therapy go beyond its prescribed usage, including cancer cells (Ben-Sahra

et al., 2011; Dowling *et al*., 2011; Pernicova *et al*., 2014; Rosilio *et al*., 2014) where metformin was shown to interfere with purine/pyrimidine and glutathione synthesis, upstream of AMPK (Corominas-Faja *et al*., 2012; Menendez *et al*., 2011) and in animal models it delayed aging, an effect observed in rodents (Anisimov *et al*., 2011; Martin-Montalvo *et al*., 2013) and in the nematode C. elegans (Onken *et al*., 2010). The mechanism underlying these positive effects remains unclear, although more recently it has been pointed that metformin retards aging in C. elegans by altering microbial folate and methionine metabolism (Cabreiro *et al*., 2013) and in mice, chronic metformin exposure lengthens lifespan and attenuates the deleterious effects of aging, resembling to some extent the effects of caloric restriction (Martin-Montalvo *et al*., 2013). Recently, it has been also validated metformin as a promising therapeutic target in neuron-related pathologies to treat neuropathic pain (Price *et al*., 2013).

In reproductive tissues, metformin has been used to treat anovulatory infertility in woman with the endocrine disorder, polycystic ovary syndrome (Bertoldo *et al*., 2014a; Palomba *et al*., 2006). However, knowledge is still insufficient to establish the benefit of metformin use during pregnancy (Bertoldo *et al*., 2014a; Legro, 2010), as metformin is able to cross the human placenta (Kovo *et al*., 2008) and more evidence is needed to ensure the safety of fetuses upon metformin treatment (Bertoldo *et al*., 2014a). In male reproductive function, metformin actions are less extensive studied and controversial (Bertoldo *et al*., 2014a). In one hand, negative effects have been described during development of human and mouse fetal testis exposed to metformin (Tartarin *et al*., 2012). Thus, metformin decreases testosterone production and mRNA of key factors involved in steroidogenesis in human and mouse fetal testis *in vitro*, reduces testicular size and the population of Sertoli cells *in vivo* during mice fetal development (Tartarin *et al*., 2012), suggesting an alteration of the physiological function of Sertoli cells during

spermatogenesis. The idea that metformin might cause toxic effects has been demonstrated later in adult male rats, showing an interference with normal testicular physiological processes leading to spermatogenic failure and marked histological alterations concomitant with a decrease in rat sperm count and motility (Adaramoye *et al*., 2012). However, it has also been suggested that metformin leads to an enhancement of the glycolytic flux in rat Sertoli cells (Alves *et al*., 2014).

The few studies about metformin in mature spermatozoa also render a controversial issue. Whereas metformin (at 50 μM concentration) did not induce negative effects on mouse spermatozoa quality (Bertoldo *et al*., 2014b), it causes adverse effects such as a clear decrease in sperm motility in boar (Hurtado de Llera *et al*., 2018) and reduction of testicular weight, spermatozoa count, sperm motility and increase in dead and abnormal spermatozoa in rabbits with alloxan-induced (type 1) diabetes (Naglaa *et al*., 2010). Moreover, beneficial effects of metformin in spermatozoa from different animal species have been also reported. Thus, metformin therapy for 8 weeks improves rat semen parameters such as concentration, viability, motility and abnormal morphology induced by high fat diet (Yan *et al*., 2015). Additionally, an anti-oxidant protector function has been attributed to metformin treatment during 4-8 weeks in spermatozoa from diabetic rats (Attia *et al*., 2009). Interestingly, only two studies about metformin in human spermatozoa have been performed to date and suggest an improvement in semen characteristics, although they were not performed in healthy men. In the fist study, oligo-terato-asthenozoospermic patients with metabolic syndrome treated with metformin for 6 months showed light improvement in insulin resistance, increase of serum androgen levels and slight sperm concentration and motility (Morgante *et al*., 2011). In the second study, metformin treatment for 3 months in hyperinsulinaemic men

significantly improved human spermatozoa morphology and enhanced chromatin packaging (Bosman *et al*., 2015).

Besides the potential clinical use of metformin in other pathologies, the rising numbers of diabetic individuals among younger populations points to an increase in the consumption of metformin in individuals of this age group. Therefore, it is necessary any effort to elucidate the cellular and molecular action of metformin in male reproductive cells. In this clinical context, the objective of the present work is to investigate *in vitro* metformin effects in human spermatozoa function. We consider that this study might be very relevant in terms of human fertility in those patients that potentially could be treated with metformin in the future.

2. Material and Methods

2.1. Ethical approval

The study was conducted in accordance with ethical guidelines in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki), therefore, informed and written consent was obtained from all individuals included in the study. Human semen was obtained from healthy donors, prepared and evaluated in line with the recommendations and current values of the World Health Organization (WHO) (World Health Organization 2010). The University of Extremadura Ethical Committee approved protocols.

2.2. Chemicals and sources

The spermatozoa density gradient Spermfilter® was from Gynotec (The Netherlands, EU). SWM medium was from IrvineScientific (Daimler, St. Santa Ana, CA, USA). Metformin was from Tocris Bioscience (Bristol, UK) and Rotenone was from Panreac (Castellar del Vallés, Barcelona, Spain). Live/dead spermatozoa viability kit (including both propidium iodide (PI) and SYBR-14 probes were from Molecular Probes (Leiden,

145 The Netherlands); MitoSOX Red Mitochondrial superoxide indicator and JC-1 probes
146 were from Life Technologies Ltd (Grand Island, NY, USA). Phosphotyrosine monoclonal
147 antibody (4G10 Platinum # 05-1050) was from Millipore (Billerica, MA, USA), anti
148 phospho-PKA substrate was from Cell Signaling Technology Inc. # 9624 (Beverly, MA,
149 USA) and anti α-Tubulin antibody was from Santa Cruz Biotechnology, Inc. (sc-8035,
150 Santa Cruz, CA, USA).

151 *2.3. Human semen samples*

152 Semen samples from 18 healthy donors were obtained by masturbation into a sterile
153 plastic container after 2-3 days of sexual abstinence, in line with the recommendations
154 of the WHO (World Health Organization 2010). After complete liquefaction (between 10
155 minutes and 1 hour at 37ºC with 5% of CO_2), samples were processed. The semen
156 parameters (total fluid volume, spermatozoa concentration, motility and morphology) of
157 all the samples fell within the WHO normality criteria. Two different fractions of
158 spermatozoa were separated by Spermfilter®, a silane-silica based 40%-80% density
159 gradient medium used in artificial reproduction techniques for the separation and
160 purification of highly motile human spermatozoa (Calle-Guisado *et al*., 2017). Highly
161 motile human sperm fraction was diluted in sperm washing medium, SWM®, for motility
162 and cytometry analysis.

163 *2.4. Human spermatozoa motility analysis*

164 High motility fractions of human spermatozoa were incubated (20×10^6 cells ml^{-1}) in
165 SWM in the absence or presence of metformin (10 mM) or rotenone (10 µM) for
166 different times (8 and 20 h) at 37ºC in a CO_2 incubator. Then, 6 µl of sample were
167 placed in a pre-warmed Spermtrack sperm counting chamber and motility was
168 evaluated using the ISAS system, both from PROISER (Paterna, Valencia, Spain). The
169 following parameters were examined: percentages of motile and progressive

spermatozoa, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral sperm head movement (ALH), linearity coefficient (LIN), straightness coefficient (SRT), wobble movement coefficient (WOB) and beat cross of flagellum frequency (BCF), as previously described (Calle-Guisado *et al*., 2017). The total number of spermatozoa evaluated in each sample was at least 300.

2.5. Analysis of spermatozoa viability by flow cytometry

Fluorescent staining using the Live/Dead sperm viability kit including SYBR-14 and PI was performed to measure spermatozoa viability, as previously described (Calle-Guisado *et al*., 2017), followed by an analysis in the flow cytometer (ACEA NovoCyte TM; ACEA Biosciences, Inc., San Diego, CA, USA) using ACEA NovoExpress TM software. The results of viable spermatozoa were expressed as the average of the percentage of $SYBR\text{-}14^{+}$ and PI^{-} labelled cells ± standard error of the mean.

2.6. Analysis of spermatozoa mitochondrial membrane potential (ΔΨm) by flow cytometry

Mitochondrial membrane potential variations (ΔΨm) were evaluated using the specific probe JC-1 (5,5′,6,6′ –tetrachloro-1,1′,3,3′ tetraethylbenzymidazolyl carbocyanine iodine), as previously described (Calle-Guisado *et al*., 2017). Results are expressed as the average percentage of spermatozoa exhibiting high mitochondrial membrane potential ± standard error of the mean.

2.7. Analysis of spermatozoa superoxide anion production by flow cytometry

Mitochondrial superoxide anion production was measured using MitoSOX probe. MitoSOX Red reagent is oxidized by superoxide anion and exhibits red fluorescence. It is readily oxidized by superoxide anion but not by other ROS- or reactive nitrogen species–generating systems. The oxidation product becomes highly red fluorescent (580 nm) upon binding to nucleic acids. 200 µl from each sperm sample were incubated

with 0.5 µl of MitoSOX (1 mM) 15 minutes at 37ºC. The fluorescence values were collected in the channel BL2 using a 572/28 nm band pass filter. Results are expressed as the average percentage of red-stained ($MitoSOX^+$) spermatozoa ± standard error of the mean.

2.8. Analysis of protein phosphorylation in human spermatozoa by western blotting.

Human sperm lysates were prepared after isolation of human spermatozoa followed by incubation at 37ºC in the absence or presence of 10 mM metformin for 8 or 20 h. Phosphorylation state of human sperm proteins was analysed by Western blot, as described (Calle-Guisado *et al*., 2017) using as primary antibodies anti-phosphotyrosine (dilution 1:5000 v/v) and anti-phosphopeptide substrates of PKA (dilution 1:1000 v/v). As protein loading control in the SDS-PAGE, we later blotted each membrane with anti-tubulin (1:500 v/v) monoclonal antibody.

2.9. Statistical analysis

Two-way repeated measures ANOVAs have been used to compare the mean differences between groups that have been split on two within-subjects factors (treatment and time). Interactions and principal effects were analyzed. Applicability conditions were directly met or, in the cases where any assumption was violated, alternative solutions were obtained to overcome this problem. Results were considered significant when p-values were lower than 0.05. IBM SPSS 19 has been used to perform the statistical analysis.

3. Results

3.1. Effect of metformin treatment in the viability of human spermatozoa

To investigate the action of metformin in viability as an essential cell parameter indicative of sperm quality and/or toxicity, human spermatozoa were incubated at 37ºC with 10 mM metformin over 20 h period and evaluated by flow cytometry. A shorter time

of 8 h was also evaluated. We have selected this metformin concentration based on our concentration-response experiments performed on boar spermatozoa (Hurtado de Llera *et al*., 2018) because 10 mM metformin causes a clear effect in other mammalian spermatozoa motility without resulting toxic or with side effects for these cells. Additionally, a treatment of spermatozoa with rotenone (10 μM), a specific inhibitor of mitochondrial complex I, was also performed in parallel in order to compare results. As observed in Figure 1, metformin does not significantly affect human sperm viability at any time studied. Under these conditions, the inhibition of the mitochondrial complex I with rotenone does not significantly affect human sperm viability either.

3. 2. Effects of metformin treatment in motility parameters of human spermatozoa.

To investigate the effect of metformin in sperm motility *in vitro*, human spermatozoa were incubated at 37ºC with 10 mM metformin for short (8 h) and long-time (20 h) and kinetic parameters were evaluated by CASA. As observed in Figure 2A, metformin treatment causes a significant reduction of the motile spermatozoa population, that resulted time-dependent. Thus, shorter metformin treatment (8 h) significantly reduces by 24% compared with control, whereas at longer time, metformin dramatically reduces the percentage of motile spermatozoa by more than 50% (statistical significance $P<0.005$). Thus, at 20 h of metformin treatment only about 26% of human spermatozoa remains motile (Fig. 2A, black histograms) compared with almost 60% of motile spermatozoa in their absence (white histograms). Rotenone treatment exerts similar effects to metformin in the percentage of motile spermatozoa. Considering the progressivity of the sperm movement (Figure 2B), although at 8 h metformin and rotenone slightly decrease it, however, at 20 h both treatments significantly reduce (by 67% and 87%, respectively) the percentage of human motile spermatozoa exhibiting progressive movement. Similarly, metformin treatment reduces the percentage of rapid

and progressive human spermatozoa (a+b) in a time-dependent manner (Figure 2C), being statistically significant at 20 h (causing 80% reduction). Rotenone treatment also significantly reduces this motility parameter at any time evaluated.

Interestingly, we have also found a very clear and reproducible time-dependent effect of metformin in human spermatozoa when velocities are studied (Figure 3). Treatment of human spermatozoa with metformin for 20 h significantly diminished all sperm velocity analyzed: curvilinear velocity VCL (Figure 3A), the straight-line velocity VSL (Figure 3B) and the average path velocity VAP (Figure 3C).

Accordingly, metformin treatment for 20 h also leads to a significant reduction in other kinetic coefficients that define sperm motility (Table 1) such as linearity LIN and straightness STR, without any detectable effect in the wobble WOB, the beat cross frequency BCF or the amplitude of lateral head displacement, ALH.

3. 3. Effect of metformin treatment in the mitochondrial activity of human spermatozoa

As observed in Figure 4A, metformin treatment of human spermatozoa does not significantly affect the mitochondrial membrane potential at any time studied. However, under same experimental conditions, spermatozoa incubation with rotenone for 20 h significantly decreases the sperm population exhibiting high mitochondrial membrane potential (statistical significance $P<0.05$). Additionally, the ability of metformin to generate mitochondrial reactive oxygen species (ROS) was investigated in parallel evaluating the production of superoxide anion. As observed in Figure 4B, metformin treatment has no effect in the generation of mitochondrial superoxide anion in human spermatozoa at any time tested. Rotenone does not significantly affect either the production of superoxide anion from human sperm mitochondria, although a slight increase effect is observed at any time.

3. 4. Effects of metformin treatment in the intracellular signaling pathways mediated by PKA and by protein tyrosine phosphorylation in human spermatozoa.

As seen in Figure 5A, metformin treatment of human spermatozoa markedly inhibits the phosphorylation of all the visualized protein substrates downstream of PKA at any time studied. In parallel, metformin treatment of human spermatozoa also potently inhibits the tyrosine phosphorylation of sperm proteins, as observed in Figure 5B. The inhibition of tyrosine phosphorylation of sperm proteins caused by metformin is clear at 8 hours but is complete at 20 hours, where no bands can be visualized under metformin treatment.

4. Discussion

This is the first study that investigates *in vitro* metformin action in human spermatozoa and demonstrates a clear adverse effect in human spermatozoa. Thus, this biguanide molecule reduces the kinetic parameters of spermatozoa from healthy men and inhibits signalling pathways mediated by PKA and by protein tyrosine phosphorylation without affecting sperm viability, the mitochondrial membrane potential or the mitochondrial superoxide anion generation at any time studied. Therefore, discarding possible toxic or side effects, our study points to a specific adverse action of metformin on human sperm motility.

An initial and relevant issue was to demonstrate that metformin, under conditions used in this study, does not posses toxic or side effects that might compromise human spermatozoa viability. Metformin concentration in the range of mM has been selected according to published data performed in other cell types (Barreto-Torres *et al*., 2015; Loubiere *et al*., 2017; Martin-Montalvo *et al*., 2013) and also in mammalian spermatozoa (Bertoldo *et al*., 2014b, Hurtado de Llera *et al*., 2018). In human spermatozoa, metformin treatment does not affect cell viability, which is in agreement

with results obtained in spermatozoa from animal species such as mouse (Bertoldo *et al*., 2014b), stallion (Cordova *et al*., 2014) or boar (Hurtado de Llera *et al*., 2018). Accordingly, in human spermatozoa, only one study has evaluated sperm viability in hyperinsulinaemic men, showing that *in vivo* metformin does not affect human sperm viability (Bosman *et al*., 2015).

The present work shows that metformin added to human spermatozoa *in vitro* causes a clear and significant reduction in sperm motility, an adverse effect that has been also reported in spermatozoa from mouse (Bertoldo *et al*., 2014b), rabbits with alloxan-induced diabetes (Naglaa *et al*., 2010) and recently in boar (Hurtado de Llera, *et al*., 2018). However, in despite of the few studies about metformin in spermatozoa, results are controversial, as it has been also reported a beneficial effect of metformin improving motility in spermatozoa from rats subjected to high-fat diet (Yan *et al*., 2015). Possible explanations to these discrepancies might be due to different maturation state of spermatozoa from distinct animal species studied (obtained from epididymis versus mature or ejaculated spermatozoa) and/or the health condition of animal studied (normal versus experimentally-induced pathology). This detrimental effect of metformin in human sperm motility is accompanied with a marked reduction in all spermatozoa velocities. Interestingly, metformin administered *in vivo* does not affect human sperm motility in hyperinsulinemic men after 3 months treatment (Bosman *et al*., 2015) or it causes a very slight increase in sperm motility after 6 months treatment (Morgante *et al*., 2011). These discrepancies with our findings may be attributed to several important differences between both studies: a) our study has been performed in spermatozoa from healthy donors, b) we added metformin *in vitro* to healthy human spermatozoa and c) in our study sperm motility was objectively evaluated by CASA system versus a subjective light microscopy analysis. It is worth mention that *in vitro* metformin also

reduces quality of the human sperm motility, causing that those human spermatozoa that remain motile after metformin treatment exhibit a clearly less progressive movement together with a less efficient motility, which results in an almost absence of rapid and progressive human spermatozoa (a+b) in the presence of metformin. Metformin effects reducing the quality of sperm motility cannot be discussed with other studies, as the two works performed to date about in vivo metformin in human spermatozoa do not evaluate any parameters that define sperm motility (Bosman *et al*., 2015; Morgante *et al*., 2011). Thus, summarizing all sperm kinetics data, we can conclude that in vitro metformin treatment leads to a drastic reduction in human spermatozoa motility.

Metformin has been described as indirect activator of AMP-activating kinase (AMPK), although the molecular mechanisms underlying this metformin action have not been elucidated yet. We demonstrated that AMPK is essential to maintain the proper sperm motility adequate to fluctuating extracellular media within the female reproductive tract in mammalian spermatozoa from boar (Hurtado de Llera *et al*., 2012; Hurtado de Llera *et al*., 2015; Hurtado de Llera *et al*., 2016) and human (Calle-Guisado *et al*., 2016; Calle-Guisado *et al*., 2017). Therefore, it is logical to assume that the metformin effect in human sperm motility might be, at least partially, attributed to its AMPK activating action. However, this is not likely the explanation, as we have not been able to show that metformin increases AMPK phosphorylation in human spermatozoa in our conditions (data not shown). This is in agreement with metformin effect in boar spermatozoa (Hurtado de Llera *et al*., 2018) and with established actions of metformin by AMPK-independent mechanisms in several cell types (Rosilio *et al*., 2014), such as cultured heart-derived cells (Saeedi *et al*., 2008), mouse embryonic fibroblasts (Ben-Sahra *et al*., 2011), acute myeloid leukemia cells (Scotland *et al*., 2013) or breast cancer cells

(Corominas-Faja *et al*., 2012) where metformin interferes with purine/pyrimidine and glutathione synthesis, upstream of AMPK. Thus, the molecular mechanisms induced by metformin appear to vary depending on the cellular context (Rosilio *et al*., 2014).

A possible explanation for metformin effect in human sperm motility includes the mitochondria activity as one of the major spermatozoa sources of oxidative energy through ATP production. It has been demonstrated that metformin causes an inhibition of the mitochondrial complex I, NADH: ubiquinone oxidoreductase, in hepatic cells (El-Mir *et al*., 2000; Owen *et al*., 2000) and cardiomyocytes (Barreto-Torres *et al*., 2015), as well as inhibition of mitochondrial respiration in skeletal muscle cells of obese rats (Kane *et al*., 2010) with obvious consequences in cell energy production. However, in human spermatozoa, metformin does not likely act through inhibition of the mitochondrial complex I, as the membrane potential ($\Delta\Psi m$) and the superoxide anion generation in the mitochondria are not affected at all by this biguanide. In agreement with this observation, it has been also reported in human muscle cells a preservation of mitochondrial complex I activity after long-term metformin treatment (Larsen *et al*., 2012). Interestingly, a study reported differential effects of metformin *in vitro* and *in vivo* in the mitochondrial complexes activity (Martin-Montalvo *et al*., 2013). Whereas in mouse embryonic fibroblasts (MEFs) metformin lowers the activity of I, III and IV mitochondrial complexes, in mice the treatment with metformin had a remarkable increase in hepatic complex I activity and remained unchanged the activity of complexes III and IV (Martin-Montalvo *et al*., 2013). In spermatozoa, metformin treatment causes a decrease in the mitochondrial membrane potential in mouse (Bertoldo *et al*., 2014b) and a total inhibition in boar (Hurtado de Llera *et al*., 2018). This effect in mouse and boar spermatozoa contrasts with the lack of effect in human spermatozoa and points to the idea that metformin action in the mitochondria activity is

likely dependent on the animal specie, besides the cell type. The idea that the sperm mitochondria is not likely responsible for the human sperm motility inhibition triggered by metformin is also supported by rotenone data. This classical well-known inhibitor of the mitochondrial complex I, which in the same experimental conditions causes a reduction in human spermatozoa motility in a very similar extent to metformin, effectively inhibits the human ΔΨm. This rotenone effect in the ΔΨm is specific, as it does not affect human spermatozoa viability. Thus, evidence in this work suggests that, unlike rotenone, the inhibition of human sperm motility caused by metformin is not likely mediated by an inhibitory action of metformin in the mitochondrial activity. In this regard, our results in human spermatozoa would be in accordance with the established proposal that metformin effects cannot be only due to inhibition of mitochondrial energy production (Hoek, 2006).

The fact that metformin does not affect the production of mitochondrial superoxide anion in human spermatozoa contrasts with the unique work performed in spermatozoa where an anti-oxidant protector function has been attributed to *in vivo* metformin treatment in diabetic rats (Attia *et al*., 2009), reducing the levels of sperm malondialdehyde and increasing sperm glutathione levels. Discrepancies can be attributed to the different species studied, distinct oxidative marker evaluated, different ways of metformin administration and to the fact that this later is performed in diabetic animals. In somatic cells, metformin might also exert antioxidative effects, although it depends on the oxidative stress stimuli (Algire *et al*., 2012; Barreto-Torres *et al*., 2015). In cardiomyocytes, metformin attenuates H_2O_2-induced ROS generation and improves the H_2O_2-induced decrease in the mitochondrial membrane potential (Barreto-Torres *et al*., 2015). In fibroblasts, metformin also attenuates paraquat-triggered ROS elevation, however has not effect in H_2O_2-induced ROS (Algire *et al*., 2012). Differences with our

finding can be explained by several reasons: i) our study is done in spermatozoa versus somatic cells; ii) metformin effects in somatic cells are referred to total cellular ROS levels, whereas we have specifically measured superoxide anion produced in the sperm mitochondria; iii) metformin effects in somatic cells are produced in response to oxidative stress inducers, whereas metformin effect in human spermatozoa is evaluated in the absence of stress inducers; iv) the metformin action in cardiomyocytes was accompanied to an improvement of the H_2O_2-reduced $\Delta\Psi m$ [44]. In human spermatozoa none of these mitochondrial parameters are affected by *in vitro* metformin, indicating that metformin acts through alternative molecular mechanisms, probably exerting different modifications in the cell metabolism according to the specific cell type. In fact, our results show that metformin treatment causes a rapid and potent inhibition of essential transduction pathways that regulate spermatozoa function, such as PKA and also signalling those involving sperm protein tyrosine phosphorylation. In this regard, it has been demonstrated that metformin treatment decreases the phosphorylation state of proteins in distinct somatic cells types that belong to different signalling pathways such as ERK in MEFs cells (Algire *et al*., 2012), α-synuclein in substantia nigra cells from mice (Katila *et al*., 2017), Akt and members of the EFGR family, HER2 and HER3, in MCF-7 and TR MCF-7 cells (Kim *et al*., 2016). Interestingly, metformin also induces a significant inhibition of protein phosphorylation downstream of PKA in both basal and stimulated conditions in B16-F10 melanoma cells by inhibiting the cAMP production (Lehraiki *et al*., 2014). In addition to cancer cells, metformin also decreases cAMP accumulation in hepatocytes, antagonizing glucagon action (Miller *et al*., 2013). Therefore, it is not surprising that metformin inhibits protein phosphorylation downstream of PKA also in human spermatozoa as we demonstrate here, likely by inhibiting the cAMP production.

Considering our results, it is becoming clear that the adverse effect of metformin on human sperm motility is likely due to the inhibition of the PKA pathway and signalling involving protein tyrosine phosphorylation, the most preponderant pathways involved in the regulation of a proper spermatozoa function.

Despite the well-known link between diabetes and male reproductive dysfunction (Tavares *et al*., 2018), and to the rising prevalence of diabetes mellitus in younger population, which involves a high percentage of males in reproductive age exposed to the chronic metformin treatment (Ferreira *et al*., 2015), the influence of metformin on this male reproductive dysfunction has been barely investigated. Metformin is currently a leading anti-diabetic drug to treat the worldwide pathology of diabetes mellitus, and given its anti-inflammatory (Martin-Montalvo *et al*., 2013) and anti-cancer (Hardie *et al*., 2014) effects, metformin could be widely used in the future to clinically treat other pathologies.

In conclusion, this work demonstrates undesirable effects of metformin at the human reproductive level: reduction of spermatozoa motility and inhibition of essential signalling pathways for the correct function of these cells. In this scientific or clinical context, the present work, that demonstrates the detrimental effect of metformin in human sperm motility, might be very relevant in terms of human fertility in those patients that potentially could be treated with metformin in the future.

Conflict of interest

Authors declare that they have no conflict of interest.

Acknowledgements

We are very thankful to donors of the semen samples. V. Calle-Guisado is recipient of a PhD fellowship (Ref. FPU14/03449) from the Ministry of Education, Culture and Sport

(Spain). This work was supported by the following Grants: AGL2015-73249-JIN, JUEX-IB16184, JUEX-IB13121 and GR10156.

Authors' roles

V.C-G performed majority of experiments, analysed the data and contributed to the draft of the manuscript. L.G-F performed western blot experiments, discussed the data and critically revised the paper. D. M-H analysed the data, discussed and critically revised the paper. L.G-M and M.J.B obtained funding, designed the study, analysed and discussed the results and critically wrote and revised the paper. All authors have read and approved the final version of manuscript.

5. References

Adaramoye O, Akanni O, Adesanoye O, Labo-Popoola O, and Olaremi O, (2012). Evaluation of toxic effects of metformin hydrochloride and glibenclamide on some organs of male rats. *Niger J Physiol Sci* 27, 137-144.

Algire C, Moiseeva O, Deschenes-Simard X, Amrein L, Petruccelli L, Birman E, Viollet B, Ferbeyre G, and Pollak MN, (2012). Metformin reduces endogenous reactive oxygen species and associated DNA damage. *Cancer Prev Res (Phila)* 5, 536-543. http://dx.doi.org/10.1158/1940-6207.CAPR-11-0536

Alves M.G., Martins A.D., Vaz C.V., Correia S., Moreira P.I., Oliveira P.F., and Socorro S., (2014). Metformin and male reproduction: effects on Sertoli cell metabolism. *Br J Pharmacol* 171, 1033-1042. http://dx.doi.org/10.1111/bph.12522

Anisimov VN, Berstein LM, Popovich IG, Zabezhinski MA, Egormin PA, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Kovalenko IG, and Poroshina TE, (2011). If started early in life, metformin treatment increases life span and postpones tumors in female SHR mice. *Aging (Albany NY)* 3, 148-157. http://dx.doi.org/10.18632/aging.100273

Attia SM, Helal GK, and Alhaider AA, (2009). Assessment of genomic instability in normal and diabetic rats treated with metformin. *Chem Biol Interact* 180, 296-304. http://dx.doi.org/10.1016/j.cbi.2009.03.001

Barreto-Torres G, Hernandez JS, Jang S, Rodriguez-Munoz AR, Torres-Ramos CA, Basnakian AG, and Javadov S, (2015). The beneficial effects of AMP kinase activation against oxidative stress are associated with prevention of PPARalpha-cyclophilin D interaction in cardiomyocytes. *Am J Physiol Heart Circ Physiol* 308, H749-H758. http://dx.doi.org/10.1152/ajpheart.00414.2014

478 Ben-Sahra I, Regazzetti C, Robert G, Laurent K, Le Marchand-Brustel Y, Auberger P,
479 Tanti JF, Giorgetti-Peraldi S, and Bost F, (2011). Metformin, independent of AMPK,
480 induces mTOR inhibition and cell-cycle arrest through REDD1. *Cancer Res* 71, 4366-
481 4372. http://dx.doi.org/10.1158/0008-5472.CAN-10-1769
482 Bertoldo MJ, Faure M, Dupont J, and Froment P., (2014a). Impact of metformin on
483 reproductive tissues: an overview from gametogenesis to gestation. *Ann Transl Med*
484 2(6), 55. http://dx.doi.org/10.3978/j.issn.2305-5839.2014.06.04
485 Bertoldo MJ, Guibert E, Tartarin P, Guillory V, and Froment P, (2014b). Effect of
486 metformin on the fertilizing ability of mouse spermatozoa. *Cryobiology* 68, 262-268.
487 http://dx.doi.org/10.1016/j.cryobiol.2014.02.006
488 Bosman E, Esterhuizen AD, Rodrigues FA, Becker PJ, and Hoffmann WA, (2015).
489 Effect of metformin therapy and dietary supplements on semen parameters in
490 hyperinsulinaemic males. *Andrologia* 47, 974-979.
491 http://dx.doi.org/10.1111/and.12366
492 Cabreiro F, Au C, Leung KY, Vergara-Irigaray N, Cocheme HM, Noori T, Weinkove D,
493 Schuster E, Greene ND, and Gems D, (2013). Metformin retards aging in C. elegans
494 by altering microbial folate and methionine metabolism. *Cell* 153, 228-239.
495 http://dx.doi.org/10.1016/j.cell.2013.02.035
496 Calle-Guisado V, Hurtado de Llera A, Gonzalez-Fernandez L, Bragado MJ, and Garcia-
497 Marin LJ, (2017). Human sperm motility is downregulated by the AMPK activator
498 A769662. *Andrology* 5, 1131-1140. http://dx.doi.org/10.1111/andr.12423
499 Calle-Guisado V, Hurtado de Llera A, Martin-Hidalgo D, Mijares J, Gil MC, Alvarez IS,
500 Bragado MJ, and Garcia-Marin LJ, (2017). AMP-activated kinase in human
501 spermatozoa: identification, intracellular localization, and key function in the

regulation of sperm motility. *Asian J Androl* 19(6), 707-714. http://dx.doi.org/10.4103/1008-682X.185848

Cho NH (chair), (2017). IDF Diabetes Atlas. 8th ed. International Diabetes Federation.

Cordova A, Strobel P, Vallejo A, Valenzuela P, Ulloa O, Burgos RA, Menarim B, Rodriguez-Gil JE, Ratto M, and Ramirez-Reveco A, (2014). Use of hypometabolic TRIS extenders and high cooling rate refrigeration for cryopreservation of stallion sperm: presence and sensitivity of 5' AMP-activated protein kinase (AMPK). *Cryobiology* 69, 473-481. http://dx.doi.org/10.1016/j.cryobiol.2014.10.008

Corominas-Faja B, Quirantes-Pine R, Oliveras-Ferraros C, Vazquez-Martin A, Cufi S, Martin-Castillo B, Micol V, Joven J, Segura-Carretero A, and Menendez JA, (2012). Metabolomic fingerprint reveals that metformin impairs one-carbon metabolism in a manner similar to the antifolate class of chemotherapy drugs. *Aging (Albany NY)* 4, 480-498. http://dx.doi.org/10.18632/aging.100472

Coughlan K.A., Valentine R.J., Ruderman N.B., and Saha A.K. (2014). AMPK activation: a therapeutic target for type 2 diabetes? *Diabetes Metab Syndr Obes* 7, 241-253. http://dx.doi.org/10.2147/DMSO.S43731

Dowling RJ, Goodwin PJ, and Stambolic V, (2011). Understanding the benefit of metformin use in cancer treatment. *BMC Med* 9, 33. http://dx.doi.org/10.1186/1741-7015-9-33

El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, and Leverve X, (2000). Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem* 275, 223-228. http://dx.doi.org/10.1074/jbc.275.1.223

525 Ferreira C, Sousa M, Rabaca A, Oliveira PF, Alves MG, and Sa R, (2015). Impact of
526 Metformin on Male Reproduction. *Curr Pharm Des* 21, 3621-3633.
527 http://dx.doi.org/10.2174/1381612821666150710150041
528 Hardie D.G., and Alessi D.R., (2013). LKB1 and AMPK and the cancer-metabolism link -
529 ten years after. *BMC Biol* 11, 36. http://dx.doi.org/10.1186/1741-7007-11-36
530 Hardie DG, and Ashford ML, (2014). AMPK: regulating energy balance at the cellular
531 and whole body levels. *Physiology* (Bethesda) 29, 99-107.
532 http://dx.doi.org/10.1152/physiol.00050.2013
533 Hardie DG, Hawley SA, and Scott JW, (2006). AMP-activated protein kinase--
534 development of the energy sensor concept. *J Physiol* 574, 7-15.
535 http://dx.doi.org/10.1113/jphysiol.2006.108944
536 Hoek J.B., (2006). Metformin and the fate of fat. *Gastroenterology* 130, 2234-2237.
537 http://dx.doi.org/org/10.1053/j.gastro.2006.05.009
538 Hurtado de Llera A, Martin-Hidalgo D, Garcia-Marin LJ, and Bragado MJ, (2018).
539 Metformin blocks mitochondrial membrane potential and inhibits sperm motility in
540 fresh and refrigerated boar spermatozoa. *Reprod Domest Anim* 53, 733-74.
541 http://dx.doi.org/10.1111/rda.13164
542 Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ, and Bragado MJ,
543 (2012). AMP-Activated Kinase AMPK Is Expressed in Boar Spermatozoa and
544 Regulates Motility. *PLoS One* 7, e38840.
545 http://dx.doi.org/10.1371/journal.pone.0038840
546 Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ, and Bragado MJ,
547 (2015). AMPK up-activation reduces motility and regulates other functions of boar
548 spermatozoa. *Mol Hum Reprod* 21, 31-45. http://dx.doi.org/10.1093/molehr/gau091

549 Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ, and Bragado MJ,
550 (2016). New insights into transduction pathways that regulate boar sperm function.
551 *Theriogenology* 85, 12-20. http://dx.doi.org/10.1016/j.theriogenology.2015.05.008
552 Kane DA, Anderson EJ, Price JW, III, Woodlief TL, Lin CT, Bikman BT, Cortright RN,
553 and Neufer PD, (2010). Metformin selectively attenuates mitochondrial H2O2
554 emission without affecting respiratory capacity in skeletal muscle of obese rats. *Free*
555 *Radic Biol Med* 49, 1082-1087.
556 http://dx.doi.org/10.1016/j.freeradbiomed.2010.06.022
557 Katila N, Bhurtel S, Shadfar S, Srivastav S, Neupane S, Ojha U, Jeong GS, and Choi
558 DY, (2017). Metformin lowers α-synuclein phosphorylation and upregulates
559 neurotrophic factor in the MPTP mouse model of Parkinson's disease.
560 *Neuropharmacology* 125, 396-407.
561 http://dx.doi.org/10.1016/j.neuropharm.2017.08.015
562 Kelly B, Tannahill GM, Murphy MP, and O'Neill LA, (2015). Metformin Inhibits the
563 Production of Reactive Oxygen Species from NADH:Ubiquinone Oxidoreductase to
564 Limit Induction of Interleukin-1beta (IL-1beta) and Boosts Interleukin-10 (IL-10) in
565 Lipopolysaccharide (LPS)-activated Macrophages. *J Biol Chem* 290, 20348-20359.
566 http://dx.doi.org/10.1074/jbc.M115.662114
567 Kim J, Lee J, Kim C, Choi J, and Kim A, (2016). Anti-cancer effect of metformin by
568 suppressing signaling pathway of HER2 and HER3 in tamoxifen-resistant breast
569 cancer cells. *Tumor Biology* 37, 5811-5819. http://dx.doi.org/10.1007/s13277-015-
570 4440-9
571 Kovo M., Haroutiunian S., Feldman N., Hoffman A., and Glezerman M., (2008).
572 Determination of metformin transfer across the human placenta using a dually

perfused ex vivo placental cotyledon model. *Eur J Obstet Gynecol Reprod Biol* 136, 29-33. http://dx.doi.org/10.1016/j.ejogrb.2007.01.013

Larsen S, Rabol R, Hansen CN, Madsbad S, Helge JW, and Dela F, (2012). Metformin-treated patients with type 2 diabetes have normal mitochondrial complex I respiration. *Diabetologia* 55, 443-449. http://dx.doi.org/10.1007/s00125-011-2340-0

Lee J.O., Lee S.K., Kim J.H., Kim N., You G.Y., Moon J.W., Kim S.J., Park S.H., and Kim H.S., (2012). Metformin regulates glucose transporter 4 (GLUT4) translocation through AMP-activated protein kinase (AMPK)-mediated Cbl/CAP signaling in 3T3-L1 preadipocyte cells. *J Biol Chem* 287, 44121-44129. http://dx.doi.org/http://dx.doi.org/10.1074/jbc.M112.361386.

Legro RS, (2010). Metformin during Pregnancy in Polycystic Ovary Syndrome: Another Vitamin Bites the Dust. *J Clin Endocrinol Metab* 95, 5199-5202. http://dx.doi.org/10.1210/jc.2010-2301

Lehraiki A, Abbe P, Cerezo M, Rouaud F, Regazzetti C, Chignon-Sicard B, Passeron T, Bertolotto C, Ballotti R, and Rocchi S, (2014). Inhibition of Melanogenesis by the Antidiabetic Metformin. *J Invest Dermatol* 134, 2589–2597. http://dx.doi.org/10.1038/jid.2014.202

Loubiere C, Clavel S, Gilleron J, Harisseh R, Fauconnier J, Ben-Sahra I, Kaminski L, Laurent K, Herkenne S, Lacas-Gervais S, Ambrosetti D, Alcor D, Rocchi S, Cormont M, Michiels JF, Mari B, Mazure NM, Scorrano L, Lacampagne A, Gharib A, Tanti JF, and Bost F, (2017). The energy disruptor metformin targets mitochondrial integrity via modification of calcium flux in cancer cells. *Sci Rep* 7, 5040. http://dx.doi.org/10.1038/s41598-017-05052-2

Martin-Montalvo A., Mercken E.M., Mitchell S.J., Palacios H.H., Mote P.L., Scheibye-Knudsen M., Gomes A.P., Ward T.M., Minor R.K., Blouin M.J., Schwab M., Pollak M.,

598 Zhang Y., Yu Y., Becker K.G., Bohr V.A., Ingram D.K., Sinclair D.A., Wolf N.S.,
599 Spindler S.R., Bernier M., and de Cabo R., (2013). Metformin improves healthspan
600 and lifespan in mice. *Nat Commun* 4, 2192. http://dx.doi.org/10.1038/ncomms3192
601 Menendez JA, Cufi S, Oliveras-Ferraros C, Martin-Castillo B, Joven J, Vellon L, and
602 Vazquez-Martin A, (2011). Metformin and the ATM DNA damage response (DDR),
603 accelerating the onset of stress-induced senescence to boost protection against
604 cancer. *Aging (Albany NY)* 3, 1063-1077. http://dx.doi.org/10.18632/aging.100407
605 Miller R.A., and Birnbaum M.J., (2010). An energetic tale of AMPK-independent effects
606 of metformin. *J Clin Invest* 120, 2267-2270. http://dx.doi.org/10.1172/JCI43661
607 Miller RA, Chu Q, Xie J, Foretz M, Viollet B, and Birnbaum MJ, (2013). Biguanides
608 suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature*
609 494, 256–60. http://dx.doi.org/10.1038/nature11808
610 Morgante G, Tosti C, Orvieto R, Musacchio MC, Piomboni P, and De Leo V. (2011).
611 Metformin improves semen characteristics of oligo-terato-asthenozoospermic men
612 with metabolic syndrome. *Fertil Steril* 95: 2150-2152.
613 http://dx.doi.org/10.1016/j.fertnstert.2010.12.009
614 Naglaa ZHE, Hesham AM, Hosny AF, and Abdel Motal SM, (2010). Impact of metformin
615 on immunity and male fertility in rabbits with alloxan-induced diabetes. *J Am Sci* 6,
616 417-426.
617 Onken B., and Driscoll M., (2010). Metformin Induces a Dietary Restriction-Like State
618 and the Oxidative Stress Response to Extend C. elegans Healthspan via AMPK,
619 LKB1, and SKN-1. *PLoS One* 5, e8758.
620 http://dx.doi.org/10.1371/journal.pone.0008758

621 Ouyang J, Parakhia RA, and Ochs RS, (2011). Metformin activates AMP kinase through
622 inhibition of AMP deaminase. *J Biol Chem* 286, 1-11.
623 http://dx.doi.org/10.1074/jbc.M110.121806
624 Owen M.R., Doran E., and Halestrap A.P., (2000). Evidence that metformin exerts its
625 anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory
626 chain. *Biochem J* 348 Pt 3, 607-614. http://dx.doi.org/10.1042/bj3480607
627 Palomba S, Orio F, Jr., Falbo A, Russo T, Tolino A, and Zullo F., (2006). Effects of
628 metformin and clomiphene citrate on ovarian vascularity in patients with polycystic
629 ovary syndrome. *Fertil Steril* 86, 1694-1701.
630 http://dx.doi.org/10.1016/j.fertnstert.2006.05.035
631 Pernicova I., and Korbonits M., (2014). Metformin--mode of action and clinical
632 implications for diabetes and cancer. *Nat Rev Endocrinol* 10, 143-156.
633 http://dx.doi.org/10.1038/nrendo.2013.256
634 Price T.J., and Dussor G., (2013). AMPK: An emerging target for modification of injury-
635 induced pain plasticity. *Neurosci Lett* 557 Pt A, 9-18.
636 http://dx.doi.org/10.1016/j.neulet.2013.06.060
637 Rosilio C., Ben-Sahra I., Bost F., and Peyron J.F., (2014). Metformin: a metabolic
638 disruptor and anti-diabetic drug to target human leukemia. *Cancer Lett* 346, 188-196.
639 http://dx.doi.org/10.1016/j.canlet.2014.01.006
640 Saeedi R, Parsons HL, Wambolt RB, Paulson K, Sharma V, Dyck JR, Brownsey RW,
641 and Allard MF, (2008). Metabolic actions of metformin in the heart can occur by
642 AMPK-independent mechanisms. *Am J Physiol Heart Circ Physiol* 294, H2497-
643 H2506. http://dx.doi.org/10.1152/ajpheart.00873.2007
644 Scotland S, Saland E, Skuli N, de TF, Boutzen H, Micklow E, Senegas I, Peyraud R,
645 Peyriga L, Theodoro F, Dumon E, Martineau Y, Danet-Desnoyers G, Bono F, Rocher

C, Levade T, Manenti S, Junot C, Portais JC, Alet N, Recher C, Selak MA, Carroll M, and Sarry JE, (2013). Mitochondrial energetic and AKT status mediate metabolic effects and apoptosis of metformin in human leukemic cells. *Leukemia* 27, 2129-2138. http://dx.doi.org/10.1038/leu.2013.107

Tartarin P, Moison D, Guibert E, Dupont J, Habert R, Rouiller-Fabre V, Frydman N, Pozzi S, Frydman R, Lecureuil C, and Froment P., (2012). Metformin exposure affects human and mouse fetal testicular cells. *Hum Reprod.* 27, 3304-14. http://dx.doi.org/10.1093/humrep/des264

Tavares RS, Escada-Rebelo S, Silva AF, Sousa MI, Ramalho-Santos J, and Amaral S, (2018). Antidiabetic therapies and male reproductive function: where do we stand? *Reproduction* 155, R13-R37. http://dx.doi.org/10.1530/REP-17-0390.

World Health Organization, (2010). WHO laboratory manual for the examination and processing of human semen. 5th edn. WHO Press, Geneva, CH.

Yan WJ, Mu Y, Yu N, Yi TL, Zhang Y, Pang XL, Cheng D, and Yang J, (2015). Protective effects of metformin on reproductive function in obese male rats induced by high-fat diet. *J Assist Reprod Genet* 32, 1097-1104. http://dx.doi.org/10.1007/s10815-015-0506-2

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, and Moller DE, (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108, 1167-1174. http://dx.doi.org/10.1172/JCI13505

668 **Figure Legends**

669 **Figure 1. Effect of metformin in human spermatozoa viability**. After isolation by
670 density gradient, human spermatozoa were incubated at 37ºC in the absence (white
671 histograms) or presence of metformin (10 mM, black histograms) or rotenone (10 µM,
672 grey histograms) for indicated times (8-20 h). Sperm viability was measured by flow
673 cytometry using SYBR-14 and PI as probes. Representative two-dimensional SYBR-14
674 fluorescence versus PI fluorescence dot plots for sperm samples incubated in presence
675 or absence of metformin or rotenone for the maximum time (20 h) are shown at the right
676 side. This experiment was performed 9 times and results, which are expressed as
677 percentage of total sperm cells analyzed, are shown as the mean ± standard error of
678 the mean (SEM). Statistical differences were not found.

679 **Figure 2. Effect of metformin in mitochondrial membrane potential (A) and**
680 **superoxide anion production (B) in human spermatozoa**. After isolation by density
681 gradient, human spermatozoa were incubated at 37ºC in the absence (white
682 histograms) or presence of metformin (10 mM, black histograms) or rotenone (10 µM,
683 grey histograms) for indicated times (8-20 h). A: Mitochondrial membrane potential
684 ($\Delta\Psi m$) was measured by flow cytometry using JC-1. Sperm population exhibiting high
685 $\Delta\Psi m$ is expressed as percentage of total sperm cells analyzed. B: Mitochondrial
686 superoxide anion production was measured using MitoSOX. Human sperm population
687 $MitoSOX^+$ is expressed as percentage of total sperm cells analyzed. Each experiment
688 was performed at least 9 times and results express the mean ± standard error of the
689 mean (SEM). Statistical differences are shown with * ($P < 0.05$).

690 **Figure 3. Effects of metformin in the percentages of motile (A), progressive (B)**
691 **and rapid progressive (C) human spermatozoa**. After isolation by density gradient,
692 human spermatozoa were incubated at 37ºC in the absence (white histograms) or

presence of metformin (10 mM, black histograms) or rotenone (10 μM, grey histograms) for indicated times (8-20 h). Following human sperm motility parameters were evaluated by ISAS system: percentage of motile spermatozoa (A), motile spermatozoa with progressive motility (B), and rapid and progressive (a+b) spermatozoa (C). This experiment was performed at least 9 times and results express the mean of the percentages of total sperm ± standard error of the mean. Statistical differences are shown with * ($P < 0.05$).

Figure 4. Effects of metformin in human spermatozoa velocities: curvilinear VCL (A), straight-line VSL (B) and average path VAP (C). After isolation by density gradient, human spermatozoa were incubated at 37ºC in the absence (white histograms) or presence of metformin (10 mM, black histograms) or rotenone (10 μM, grey histograms) for indicated times (8-20 h). Following sperm motility parameters were evaluated by ISAS system: curvilinear velocity VCL (A), straight-line velocity VSL (B) and average velocity VAP (C), expressed as μm/s. This experiment was performed at least 9 times and results express the mean of the percentages of total sperm ± standard error of the mean (SEM). Statistical differences are shown with * ($P < 0.05$).

Figure 5. Effect of metformin in the phosphorylation of proteins in human spermatozoa. After isolation by density gradient, human spermatozoa were incubated at 37ºC in the absence or presence of metformin (10 mM) for indicated times (8-20 h) and cell lysates were prepared. A: Phosphorylation state of sperm proteins downstream of PKA was evaluated using a specific antibody. B: Tyrosine phosphorylated protein pattern was analyzed by western blot with anti-phosphotyrosine as primary antibody (upper film). Loading control was performed with anti-tubulin antibody (lower film).

Table 1. Effects of metformin in human spermatozoa motility coefficients. After isolation by density gradient, human spermatozoa were incubated at 37ºC in the

718 absence or presence of metformin (10 mM) or rotenone (10 μM) for indicated times (8-
719 20 h). Following coefficients that characterize human sperm motility were evaluated by
720 ISAS system: LIN: linearity; SRT: straightness; WOB: wobble movement coefficient;
721 BCF: beat cross frequency and ALH: amplitude of lateral sperm head displacement.
722 This experiment was performed at least 9 times and values are expressed as indicated
723 and represent the mean ± SEM. Values bearing an * differ statistically from their own
724 control at the same incubation time ($P < 0.05$).

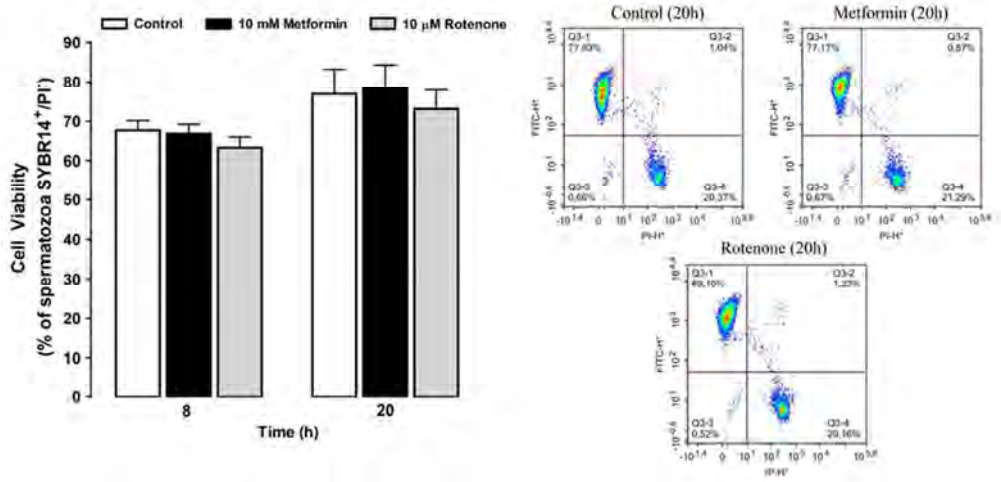

Figure 1. Effect of metformin in human spermatozoa viability.

74x36mm (300 x 300 DPI)

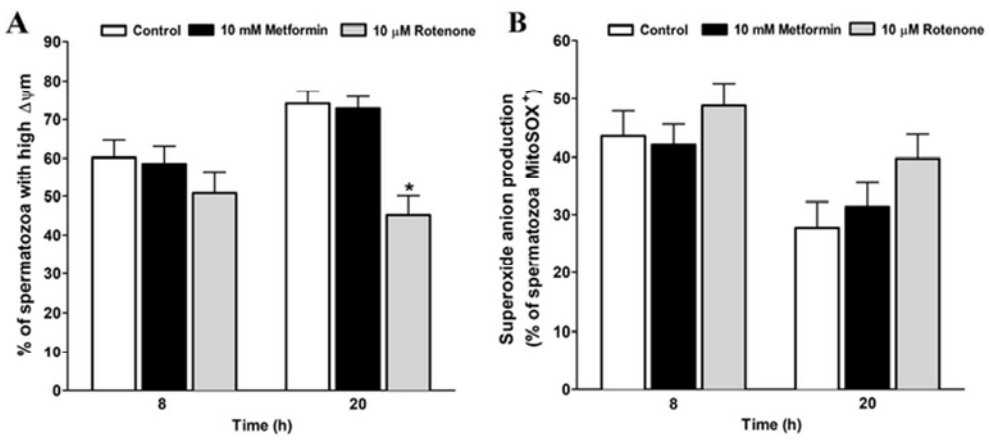

Figure 2. Effect of metformin in mitochondrial membrane potential (A) and superoxide anion production (B) in human spermatozoa.

65x28mm (300 x 300 DPI)

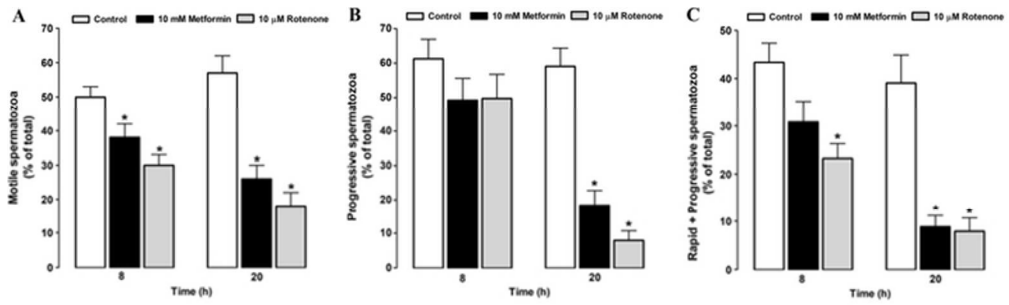

Figure 3. Effects of metformin in the percentages of motile (A), progressive (B) and rapid progressive (C) human spermatozoa.

60x18mm (300 x 300 DPI)

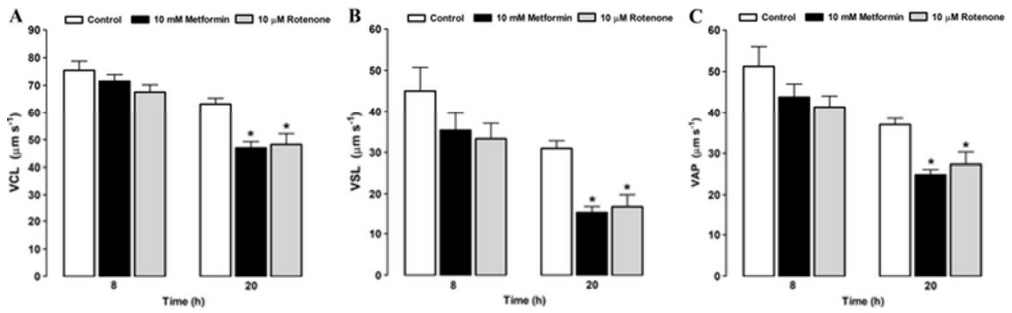

Figure 4. Effects of metformin in human spermatozoa velocities: curvilinear VCL (A), straight-line VSL (B) and average path VAP (C).

62x19mm (300 x 300 DPI)

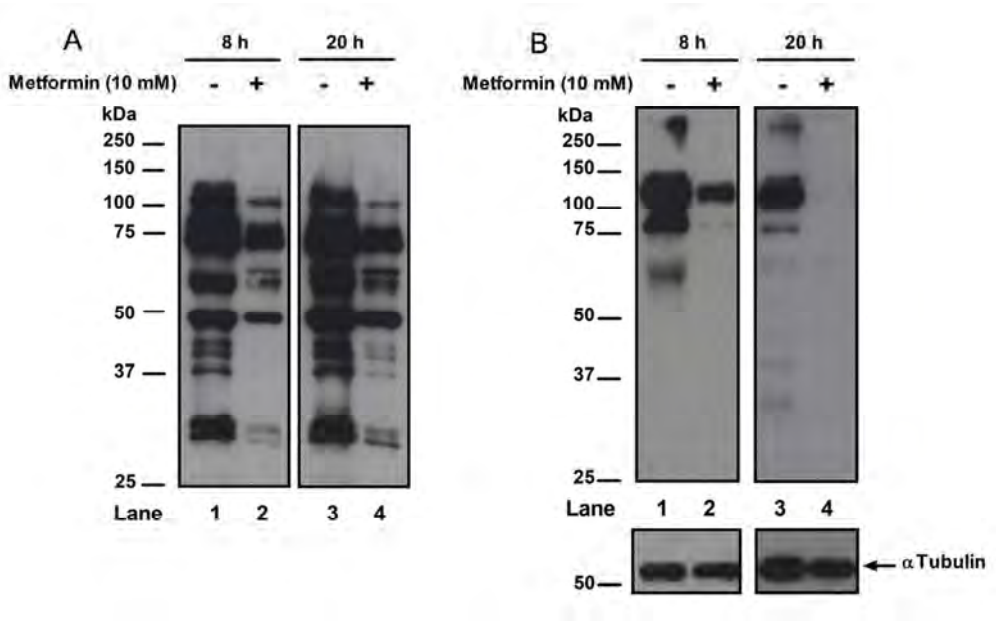

Figure 5. Effect of metformin in the phosphorylation of proteins in human spermatozoa.

92x56mm (300 x 300 DPI)

Table 1. Effects of Metformin and Rotenone in motility parameters coefficients of human spermatozoa.

	8 h			20 h		
	Control	Metformin (10 mM)	Rotenone (10 μM)	Control	Metformin (10 mM)	Rotenone (10 μM)
LIN (%)	54.7 ± 4.6	46.6 ± 4.0	46.3 ± 4.1	47.4 ± 2.5	32.2 ± 1.8*	32.8 ± 2.1*
STR (%)	78.1 ± 2.9	72.7 ± 3.3	71.3 ± 3.5	77.6 ± 2.3	59.0 ± 2.4*	56.1 ± 2.7*
WOB (%)	66.1 ± 3.2	60.5 ± 2.1	61.3 ± 2.1	58.7 ± 1.3	54.5 ± 0.7*	56.8 ± 0.6
BCF (Hz)	8.1 ± 0.4	7.9 ± 0.5	7.7 ± 0.6	7.8 ± 0.4	5.3 ± 0.3	5.3 ± 0.6
ALH (μm)	2.6 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	2.1 ± 0.1*

After isolation by density gradient human sperm were incubated at 37°C in the absence or presence of metformin (10 mM) or rotenone (10 μM) for indicated times (8-20 h). Following coefficients that characterize human sperm motility were evaluated by ISAS system: LIN: linearity; SRT: straightness; WOB: wobble movement coefficient; BCF: beat cross frequency and ALH: amplitude of lateral sperm head displacement. This experiment was performed at least 9 times and values are expressed as indicated and represent the mean ± SEM. Values bearing an * differ statistically from their own control at the same incubation time ($P < 0.05$).