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## Enzymatic activity of mouse Group X-sPLA2 improves *in vitro* production of preimplantation bovine embryos

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development

## Abstract 2,500 keystrokes

Assisted reproductive technologies (ART) are widely used for both humans and domestic animals. In bovine species, *in vitro* embryo production is increasingly used and significant efforts are being made to optimize media and culture conditions. Phospholipase A2 (PLA2) are lipolytic enzymes that hydrolyze glycerophospholipids to produce free fatty acids and lysophospholipids that have been found to be critical for many biological processes. Mouse group X secreted PLA<sub>2</sub> (mGX) is abundant in the male reproductive tract and its use during sperm capacitation has been shown to improve *in vitro* production of viable embryos in a mouse model. Here, we examined its effect in the bovine species, testing the impact of mGX on the three steps involved in *in vitro* production of preimplantation embryos: oocyte maturation, fertilization and preimplantation development. We found that incubating cumulus oocyte complexes (COC) or gametes with mGX resulted in increased blastocyst hatching and blastocyst production, respectively. The increases of embryo production induced by the phospholipase mGX were not observed for the catalytically inactive mutant H48Q-mGX, suggesting that these effects require the enzymatic activity of mGX. We also tested bGIB, a bovine homolog of mGX. bGIB failed to improve blastocyst production, underlining the high specificity of mGX. In conclusion, the results presented show that the effects of mGX are not restricted to the mouse model and that it is potent in the bovine species as well. This result strengthens the potential of mGX as a “pro-fertility drug” for mammalian reproduction.

### 1. Introduction

Assisted reproductive technologies (ART) have greatly improved in recent decades, and are now widely used in humans and to manage animal reproduction [1-3]. In the bovine species, sperm cryopreservation and artificial insemination are currently the main ART used. However, *in vitro* embryo production has emerged as an interesting technology for specific cattle breeding objectives [4]. For instance *in vitro* embryo production maximizes the use of remarkable cows to produce more embryos with females presenting outstanding genetic background. Despite continuous efforts to improve *in vitro* blastocyst production efficiency, its yield remains low, and the proportion of oocytes reaching the blastocyst stage is barely 30 to 40%, which is half the yield obtained when combining ovarian stimulation with *in vivo* fertilization. Moreover, *in vitro*-produced blastocysts appear as being of lower quality than those produced *in vivo* [3, 5-7].

60 Optimization of culture media through supplementation with hormones, proteins and growth factors is one of the main current fields of research in *in vitro* blastocyst production; its main goal is to improve both efficiency of the technique and embryo quality [8-17].

In our search to identify new compounds which improve *in vitro* production of bovine blastocysts, we assessed the impact on *in vitro* blastocyst production of treating bovine gametes and early embryos with phospholipase A<sub>2</sub> (PLA<sub>2</sub>). PLA<sub>2</sub>s form a superfamily of proteins hydrolyzing the sn2-ester bonds of glycerophospholipids, and are involved in many biological functions [18]. Three PLA<sub>2</sub> subfamilies have been defined, based on calcium-sensitivity and their biological localization: two subfamilies are intracellular and calcium-independent (cPLA<sub>2</sub> and iPLA<sub>2</sub>) whereas the third subfamily is calcium-dependent and secreted (sPLA<sub>2</sub>) [19]. sPLA<sub>2</sub> is the largest subfamily, with 12 known isoforms. All PLA<sub>2</sub> are involved in key steps of male and female reproductive physiology [20].

In males, both sPLA<sub>2</sub> and iPLA<sub>2</sub> are expressed in the testes and accessory reproductive glands. They are involved in spermatogenesis, sperm maturation and sperm acrosome exocytosis. For instance, iPLA<sub>2</sub>β deficiency leads to abnormal sperm formation and a defective acrosome reaction [21, 22]. Mouse sPLA<sub>2</sub> of group III (mGIII) is required for lipid remodeling during epididymal transit, and its deficiency results in severe epididymal maturation defects [23]. Previously, we showed that the mouse sPLA<sub>2</sub> of group X (mGX) encoded by the Pla2g10 gene is present in the acrosome of murine sperm and is secreted during the acrosome reaction, whether induced or spontaneous [24]. mGX induces changes in the lipid composition of the plasma membrane of the sperm, resulting in modified sperm mobility [25]. By a paracrine effect, mGX in conjunction with iPLA<sub>2</sub>β amplifies the acrosome reaction [22]. In mice, mGX has been shown to be involved in sperm fertilizing competence, and its absence (in knockout mice) results in a lower initial cleavage rate and a higher rate of early death in the early stages of *in vitro* development. These deleterious effects can be counteracted by adding recombinant mGX to the medium containing knockout sperm. Interestingly, treatment of normal sperm with recombinant mGX improves the production of viable murine embryos [24], opening a relevant field of investigation into the use of PLA<sub>2</sub>, and in particular mGX, in assisted reproduction in other species including bovine [26]. sPLA<sub>2</sub> have an enzymatic activity (hydrolyzation) but also induce biological responses through mechanisms requiring their binding to specific membrane receptors [27]. In the mouse model we had demonstrated that the ability of mGX to increase viable embryo production was dependent on its enzymatic activity [24].

Compared to the mouse model, the *in vitro* production of bovine embryos usually requires an additional step corresponding to *in vitro* maturation of oocytes as the female gametes are obtained by puncture of immature follicles from ovaries. In this study, we therefore investigated the impact of adding recombinant mGX, a catalytically inactive mutant of mGX and the bovine group IB sPLA<sub>2</sub> during *in vitro* oocyte maturation, fertilization and early preimplantation development of bovine embryos, on initial cleavage, production of blastocyst and blastocyst hatching rates.

## 100        2. Materials and methods

### 2.1. Semen collection

Bull semen was collected by artificial vagina at the OrigenPlus technical center (L'Aigle, France). Ejaculate was diluted 1:1 in Optixcell (IMV technologies, L'Aigle, France) and after dilution, frozen and then stored in liquid nitrogen until required for *in vitro* fertilization.

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### 2.2. *In vitro* maturation of oocytes

Bovine ovaries were collected at the slaughterhouse and transported in NaCl 0.9% supplemented with 0.2% of a solution containing 1% of kanamycin sulfate (Thermo Fisher Scientific, France) within 3 h in a container maintained at 35 °C. Cumulus-oocytes complexes (COCs) were recovered from 2-8 mm antral follicles by manual aspiration using a sterile 18-gauge needle attached to a sterile 5 mL syringe containing collection medium (1% v/v PVA (Poly(Vinyl Alcohol)); 4.2 mM NaHCO<sub>3</sub>; 10 mM HEPES; 2 mM glutamine; 50 IU/mL penicillin; 50 µg/mL streptomycin, in M199 medium). Only grade 1 and 2 COCs were used for further processing [28]. Groups of around 30 COCs were matured in 4-well plates with 500 µL oocyte maturation medium per well for 24 h at 38.5 °C under 5% CO<sub>2</sub> and saturating humidity. Oocyte maturation medium corresponded to a modified M199 medium, comprising M199 medium and Earle's salts (Life technologies®) supplemented with 2 mM glutamine, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/mL gentamycin, 10% v/v FBS (Fetal Bovine Serum) and 0.2 µg/mL EGF (Epidermal Growth Factor).

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### 2.3. *In vitro* fertilization

Semen straws were thawed in a water-bath at 37 °C for 45 s. Straws were wiped down with 70° ethanol before opening. The 0.25 mL thawed semen from each straw was layered under 4 mL of Sp-TALP capacitation medium (NaCl 114.0 mM; KCl 3.2 mM; NaH<sub>2</sub>PO<sub>4</sub> 0.3 mM; Na

125 Lactate 60% 10 mM; MgCl<sub>2</sub> 0.5 mM; HEPES 10 mM; NaHCO<sub>3</sub> 25 mM; Na Pyruvate 1 mM  
and gentamycin 50 µg/mL; adjusted to pH 7.4) supplemented with 6 mg/mL bovine serum  
albumin (BSA) and 2 µg/mL heparin) in a 15 mL conical tube. Sperm were allowed to swim-  
up at 37 °C for 1 h. At the end of the incubation period, the upper layer, containing motile  
sperm, was collected and centrifuged, and the pellet was resuspended in fertilization medium.  
130 Sperm concentration was measured and 750,000 sperm (or 450,000 when suboptimal sperm  
concentration was tested) were injected into a 500 µL droplet of fertilization medium containing  
30 COCs. The composition of *in vitro* fertilization (IVF) medium was NaCl 114.0 mM; KCl  
3.2 mM; NaH<sub>2</sub>PO<sub>4</sub> 0.3 mM; Na Lactate 10.0 mM; CaCl<sub>2</sub> 2.0 mM; MgCl<sub>2</sub> 0.5 mM; NaHCO<sub>3</sub>  
25.0 mM; Minimum Essential Medium (MEM), 50X Amino Acid solution (Life technologies)  
135 2% vol/vol; 6 mg/mL BSA; 0.2 mM pyruvate; 50 µg/mL gentamycin; 23.5 µg/mL heparin;  
20 µM/mL penicillamine; 10 µM hypotaurine and 2 µM/mL epinephrine. Sperm and COCs  
were co-cultured at 38.5 °C under 5% v/v CO<sub>2</sub> and saturating humidity for 24 h.

#### 2.4. *In vitro* embryo culture

140 Presumed zygotes were removed from fertilization wells 24 hours after fertilization, vortexed  
for 2 min to remove cumulus cells and washed three times by passing through dishes containing  
collection medium. Presumed zygotes were then cultured in 500 µL embryo culture medium in  
4-well plates. Embryo culture medium was mKSOM/aa (modified potassium-supplemented  
simplex optimised medium) supplemented with 20% RD (RPMI-1640 and DMEM, 1:1  
145 vol/vol), RPMI = Roswell Park Memorial Institute medium-1640 medium (Life technologies,  
ref 11879-020) and DMEM = Dulbecco's Modified Eagle's Medium (Sigma, ref D6046).  
mKSOM/aa contained NaCl 98.6 mM; KCl 2.5 mM; CaCl<sub>2</sub> 1.71 mM; KH<sub>2</sub>PO<sub>4</sub> 0.35 mM;  
MgSO<sub>4</sub> 0.2 mM; Na Pyruvate 0.3 mM; Na Lactate 3.0 mM; NaHCO<sub>3</sub> 25 mM; HEPES  
10.0 mM; 0.27% L-Glutamine (Life technologies 25030-032) + 1.6 % essential amino acids  
150 (Life technologies ref 11130-036) and 0.8% non essential amino acids (Life technologies, ref  
11140-035); insulin 5 µg/mL; transferrin 5 µg/mL; Na-Selenite 5 ng/mL; EDTA 10 µM,  
dibekacin sulfate 65 µg/mL [29], and covered with 250 µL equilibrated mineral oil. Embryos  
were maintained in culture for 9 days. Post-fertilization, cleavage rates were assessed at 48 h,  
blastocyst formation rate at 7 days, and hatching rate at 9 days, by microscopic observation by  
155 a trained technician and based on their morphology.

#### 2.5. Experimental design

In this study, we tested the impact of exposure to recombinant mGX and two other sPLA<sub>2</sub>s (H48Q-mGX and bovine group IB) during the following three phases: *in vitro* maturation (IVM), *in vitro* fertilization (IVF), *in vitro* development (IVD) or a combination of IVM and IVF on embryo production. For IVM, IVF or IVM+IVF treatments, we compared the rates of 2-cell embryos at 48 h post fertilization and the rates of blastocysts and hatched blastocysts at 7 and 9 days (D+7 and D+9), respectively, obtained in control conditions or following exposure to the different drugs during the period of treatment. For IVD treatment, we compared only the rates of blastocysts and hatched blastocysts at D+7 and D+9 (Fig. 1).

In details, for IVM treatment, COCs were divided in two groups, the control group was processed as explained above and the treated group was incubated in oocyte maturation medium containing 200 nM mGX for 24 h. Control and treated COCs were then rinsed twice in oocyte maturation medium and introduced into a fertilization droplet containing fertilization medium (described above) (Fig.1A). For IVF treatment, the control group of gametes (COCs and sperm) were introduced in a fertilization droplet containing fertilization medium only and the treated group of gametes in a fertilization medium supplemented with 200 nM mGX, for 24 h. Then, presumed zygotes were washed and processed as explained in “*in vitro* embryo culture” paragraph (Fig.1A). For IVD treatment, presumed zygotes were produced in control conditions and then divided in two groups. Control group was cultured in embryo culture medium (described above) and treated group in culture medium supplemented with 200 nM mGX for 8 days. For IVM+IVD treatments, the control group correspond to COC cultured and fertilized in control medium whereas treated group correspond to COC cultured and fertilized in maturation and fertilization media supplemented with either mGX (200 nM), H48Q-mGX (200 nM) or bGIB (200 nM) (Fig.1A). Finally, we tested mGX effect with a suboptimal concentration of sperm for IVF: for the control group, COC were fertilized with 450 000 sperm cells (0.9 M/mL) and for treated group, COCs were fertilized with 450 000 sperm cells (0.9 M/mL) in a fertilization medium supplemented with 200 nM mGX . The effect of mGX addition on cleavage, blastocyst and hatching rates were compared between control and treated groups (Fig. 1B)

## 2.6. Production of recombinant sPLA<sub>2</sub>

Mouse group X sPLA<sub>2</sub> and its catalytically inactive mutant H48Q-mGX were produced as described in [30]. Briefly, sPLA<sub>2</sub>s was produced in *E. coli* as an inclusion body protein, it was

refolded *in vitro*, and the resulting protein was extensively HPLC-purified. Purified sPLA<sub>2</sub> was characterized by electrophoresis and mass spectrometry, and specific enzymatic activity was measured using radiolabeled *E. coli* membranes as substrates. Bovine group IB sPLA<sub>2</sub> was purchased from Sigma Aldrich, and characterized by mass spectrometry to validate the composition of the purchased lot.

### 2.7. Chemicals

All chemicals were purchased from Sigma Aldrich unless otherwise indicated.

### 2.8. Statistical analysis

Data were treated using R software (version number 3.0.3). The percentage of 2-cell embryos, blastocysts and hatched blastocysts from control and treated groups were compared based on paired t-tests. A probability value of less than 0.05 was considered statistically significant. Data are presented as mean  $\pm$  SEM.

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### 3. Results

#### 3.1 Effects of mGX addition during IVM on cleavage, blastocyst and hatching rates

Our first objective was to assess whether incubating COCs with 200 nM mGX affected embryo production. Exposure to mGX during *in vitro* oocyte maturation had no impact on the rate of initial cleavage and the production of blastocysts (Fig. 2B,C). However, a significant increase in the yield of hatched blastocysts was observed at D+9 (Fig. 2D). The mean of hatched blastocyst percentage increased upon treatment from 39.95 to 67.93%, corresponding to a 70% increase of hatched blastocysts (n=9, p=0.00001, paired t-test). Moreover, this increasing rate of hatching is associated with a decrease of the rate of non-expanded blastocysts (Fig. 2E).

#### 3.2 Effects of mGX addition during IVF on cleavage, blastocyst and hatching rates

Our second objective was to assess whether adding mGX during fertilization process at the time of sperm injection impact embryo development. The yields of 2-cell embryos and blastocysts were both significantly increased following mGX treatment (Fig. 3BC). The mean of 2-cell embryos percentage increased upon treatment from 94.70 to 97.82%, corresponding to a 3.3% increase in 2-cell embryos (n=7, p=0.043, paired t-test). This 3.3% increase is close to the maximal possible increase of 5.6%, corresponding to an increase from 94.7% to 100% of fertilized oocytes (Fig. 3B). The percentage of increase of total blastocyst formation induced by mGX treatment reached 21.4%, from 30.17 to 36.6% (Fig. 3C). However, the rate of blastocyst hatching was not statistically different between the two conditions (Fig.3D). Interestingly, the effect of mGX treatment was considerably improved when fertilization was performed with suboptimal sperm concentrations (sperm concentration was lowered from 1.5 million/mL to 0.9 million/mL), the percentage of increase, rising from 3.3 to 42.4% for 2-cell embryos and from 21.4 to 102.5% for blastocysts, for optimal and suboptimal sperm concentration, respectively (Fig. 3EF).

#### 3.3 Effects of mGX addition during IVD on cleavage, blastocyst and hatching rates

Finally, we tested the impact of mGX on embryo development by incubating embryos with 200 nM mGX throughout embryo development (From D+1 to D+9). In these conditions, PLA<sub>2</sub> treatment was deleterious and a significant decrease in blastocyst production and blastocyst hatching was recorded (Fig. 4).

#### 3.4 Effects of mGX addition during IVM and IVF.

The results presented above show that mGX affects the production of blastocysts differently when applied during IVM and IVF steps. We next examined whether application of mGX from IMV to IVF produced an additive positive effect, increasing both the rate of blastocyst formation and blastocyst hatching. With this protocol, increased 2-cell embryo yields, 240 blastocyst formation, and blastocyst hatching rates was observed, demonstrating that the effects recorded during both steps were additive (Fig. 5).

### 3.5 Effects of catalytically-inactive mGX mutant addition during IVM and IVF

As introduced above, sPLA<sub>2</sub> have a dual effect, through enzymatic activity (hydrolyzation) and/or through mechanisms requiring their binding to specific membrane receptors. We 245 therefore evaluated whether H48Q-mGX, a catalytically inactive mGX mutant, which keeps its ability to bind to the receptor [31], was able to induce an increase of embryo production following treatment during IVM and IVF. In contrast to mGX, the mutant, failed to stimulate the production of 2-cell embryos, blastocysts or hatched blastocysts (Fig. 6).

### 250 3. Effects of the bovine bGIB sPLA<sub>2</sub> addition during IVM and IVF

Recent publications indicate that LysoPhosphatidic Acid (LPA), a byproduct of PLA<sub>2</sub> can improve *in vitro* maturation of bovine oocytes and further development [32-34]. To test the hypothesis that the effect of mGX could be that it enhances LPA production, we tested a bovine sPLA<sub>2</sub> known to efficiently produce LPA [35]. Exposure to the bovine group IB sPLA<sub>2</sub> (bGIB) 255 during oocyte maturation and fertilization steps did not reproduce the effects of exposure to mGX (Fig.7).

## 4. Discussion

The aim of this study was to assess whether sPLA<sub>2</sub> improved *in vitro* production of blastocysts 260 in the bovine model. We tested two sPLA<sub>2</sub>s, the bovine bGIB and the mouse mGX. The mouse isoform was tested because we had previously shown that mGX increased the production of viable embryos in a mouse model [24], whereas all other mouse or human sPLA<sub>2</sub>s tested failed to improve embryo production in this model [36]. We also tested the catalytically inactive mutant H48Q-mGX to investigate the mode of action of mGX. In the mouse model, we only 265 tested the effect of sPLA<sub>2</sub> treatment during the capacitation of sperm on viable embryo production. In this report, we extended our analysis, with, for the first time, tests of the effect

of sPLA<sub>2</sub> applied during the three steps required to produce bovine preimplantation embryos *in vitro*: oocyte maturation, fertilization and preimplantation development. Our results showed: first, that treatment of COC during oocyte maturation with mGX led to increased hatched  
270 blastocyst production. This effect is likely due an increase of the developmental competence of mGX-treated oocyte, since the rate of non-expanded blastocysts is lower when COCS were treated (Fig 2E). Second, we showed that treating sperm and oocytes with mGX during fertilization step had a specific effect on embryo production. Third, application of mGX from oocyte maturation to fertilization steps improve both embryo production and blastocyst  
275 hatching, which is neither mimicked by bGIB or H48Q-mGX. Finally, we demonstrated that mGX has a detrimental effect when used during preimplantation development. Moreover, by assessing the timing of full blastocyst formation and the ability to hatch, our study allows an evaluation of the impact of mGX treatment on embryo quality. Indeed, the kinetics of development is reported to be a good indicator of embryo quality for both human and bovine  
280 embryos [37, 38] along with timing of full blastocyst formation, degree of blastocyst expansion and the ability to hatch [39, 40].

#### 4.1. PLA<sub>2</sub> and oocyte maturation

Lipid metabolites are important for oocyte maturation, in particular through the production of LPA. LPA was shown to have a positive effect on oocyte maturation and subsequent  
285 fertilization competence of oocytes in rodent, bovine and porcine species [32, 41, 42] due to changes observed in cumulus cells [33, 43]. LPA synthesis first requires activation of phospholipase D (PLD) [44], which converts phosphatidylcholine to phosphatidic acid (PA), and then requires activation of a PLA<sub>2</sub> to hydrolyze the sn-2 ester bond of PA, producing LPA. Numerous PLA<sub>2</sub> have been described as involved in this process, including members of the  
290 iPLA<sub>2</sub>, cPLA<sub>2</sub> and sPLA<sub>2</sub> subfamilies. Among sPLA<sub>2</sub>, several groups can convert PA into LPA. Based on an *in vitro* assay measuring LPA production by different mouse and human sPLA<sub>2</sub> from a mixture of different phospholipids, mouse and human group X and IB sPLA<sub>2</sub> were reported to display similar potency [35]. Although both groups X and IB produce similar levels of LPA, here, we demonstrated that only mGX improves oocyte competence. This result  
295 strongly suggests that the effect of PLA<sub>2</sub> does not rely on LPA production. Group X sPLA<sub>2</sub>s are also known to release arachidonic acid from mammalian cells more effectively than other PLA<sub>2</sub>. Arachidonate is the primary substrate for prostaglandin production, and prostaglandin E2 is a key factor regulating mammalian oocyte maturation [45]. Therefore, treatment of COC by mGX probably promotes arachidonate release, triggering prostaglandin production which

300 positively regulates oocyte maturation. The fact that the enzymatically-inactive H48Q-mGX mutant has no effect supports this hypothesis. Oocyte quality is the cornerstone of reproductive success, both *in vitro* and *in vivo*, and enhanced oocyte maturation in the presence of mGX will have a positive impact on embryo development, in particular in bovine species where embryo genome activation is delayed until day 4.

#### 305 4.2. Impact of gametes treatment on embryo production

The discovery of mGX's action on the production of viable embryos was first described in the mouse model, and its action was shown to require enzymatic activity [24]. In this report, we show that mGX is also potent in the bovine model. In the mouse model, only sperm was treated and increased 2-cell embryo production. In the bovine model, both sperm and oocytes were  
310 simultaneously treated during the fertilization step and this treatment improved rates of both 2-cell embryo and blastocyst production, obtained with suboptimal and optimal sperm concentration. In suboptimal conditions, mGX is more effective, approaching the efficiency for 2-cell embryos observed in the mouse model. In optimal condition, the increase in the yield of blastocyst embryos is not due to the generation of increased numbers of 2-cell embryos, because  
315 the increase in 2-cell embryos is just 3.3%, compared to an increase in blastocysts of 21.4%. Moreover, we show that bGIB, another sPLA<sub>2</sub> has no effect. So far, thus mGX is the only sPLA<sub>2</sub> shown to improve sperm's ability to produce viable embryos. We therefore confirm that mGX has exceptional specificity [36]. Importantly, our results demonstrate that mGX is potent on at least two species, and that its efficacy is not restricted to the mouse model, even though it  
320 is a mouse isoform. Finally, the enzymatically-inactive H48Q-mGX mutant has no effect on bovine sperm, confirming that the effect observed with mGX requires production of lipid metabolites.

#### 4.3. PLA<sub>2</sub> and embryo development

The molecular mechanism behind the inhibition of development of preimplantation embryos  
325 was not explored here, but we can speculate that this effect may be due to overproduction of arachidonic acid and excessive stimulation of the prostaglandin cascade. Indeed, preimplantation embryos have the enzymes needed to synthesize prostaglandin from arachidonic acid [46], and prostaglandins are known to control the growth factor-induced mitogenic pathway [47]. Therefore, an imbalance in this pathway could lead to developmental  
330 defects.

#### 4.4. Conclusion

We have demonstrated for the first time that treating bovine gametes with sPLA<sub>2</sub> during either *in vitro* oocyte maturation or/and fertilization periods has a beneficial effect on blastocyst production, improving both their quantity and quality. In contrast, treating embryos during pre-implantation development hampers their development. The enzymatic properties of sPLA<sub>2</sub> are crucial, since beneficial effects were observed with mouse group X sPLA<sub>2</sub> whereas bovine group IB sPLA<sub>2</sub> is embryo-toxic, hampering the blastocyst hatching (Fig. 7). Therefore, efficacy of mGX is not restricted to the mouse model and confirms the potential of mGX as a “pro fertility drug” for mammalian reproduction.

340

#### Declaration of interest

A patent “Fertilization modulation compounds and process for implementing them” EP09305261. 24 March 2009) has been submitted by Université Grenoble Alpes & CNRS & INSERM

345

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Fig. legends

**Fig. 1.** Experimental protocol showing the different periods of treatment (IVM, IVF and  
355 IVM+IVF) and the timing of assessment of *in vitro* embryos production.

(A) Mouse group X (mGX) sPLA<sub>2</sub> was applied either during in vitro maturation (IVM) of COC  
for 24 h, *in vitro* fertilization (IVF) for 24 h or *in vitro* development (IVD) for 8 days (from day  
1 (D+1) to day 9 (D+9)). Mouse group X and two other sPLA<sub>2</sub>s were also applied during a  
360 combination of IVM and IVF for 48 h. At day 0 (D0), mature COC were fertilized and incubated  
together with sperm for 24 h. At day 1 (D+1) presumed zygote were transferred to culture  
medium and then cultured up to day 9 (D+9). (B) Two-cell embryos were assessed at day 2  
(D+2), blastocyst at day 7 (D+7) and hatched blastocyst at day 9 (D+9), as indicated.

**Fig. 2.** mGX-sPLA<sub>2</sub> enhances blastocyst expansion and hatching when applied during *in vitro*  
365 maturation (IVM) of oocytes.

(A) mGX (200 nM) was applied during the IVM stage only. (B) Yield of 2-cell embryos on day  
2 for COC incubated in control and mGX media versus total number of matured oocytes. (C)  
Yield of blastocysts on day 7. (D) Yield of hatched blastocysts on day 9. (E) Proportions of  
non-expanded, expanded and hatched embryos on day 9. n = 9 replicates. Range of oocytes  
370 number per condition: 215-225. Statistical differences were assessed using a paired t-test.

**Fig. 3.** mGX-sPLA<sub>2</sub> enhances 2-cell embryo and blastocyst production when applied during *in*  
*vitro* fertilization (IVF).

(A) mGX (200 nM) was applied during the IVF step only. (B) Yield of 2-cell embryos on day  
375 2 for gametes incubated in control and mGX-supplemented media. (C) Yield of blastocysts on  
day 7. (D) Yield of hatched blastocysts on day 9. n = 7 replicates. Total number of oocytes: 195  
± 5/condition (mean ± SEM). Statistical differences were assessed using a paired t-test. (E, F)  
mGX effect during IVF is improved when oocytes are fertilized with a suboptimal sperm  
concentration (sperm concentration was lowered from 1.5 million/mL to 0.9 million/mL).  
380 Marked improvement is measured for production of 2-cell embryos (E) and blastocysts (F). n

= 4 replicates. Range of oocytes number per condition: 165-175. Statistical differences were assessed using a paired t-test.

**Fig. 4.** mGX-sPLA<sub>2</sub> applied during the *in vitro* development (IVD) step reduces embryo production.

(A) mGX (200 nM) was applied during the IVD step only. (B) Yield of 2-cell embryos on day 2 for embryos incubated in control and mGX-supplemented media. (C) Yield of blastocysts on day 7. (D) Yield of hatched blastocysts on day 9. n = 3 replicates. Range of oocytes number per condition: 110-120. Statistical differences were assessed using a paired t-test.

390

**Fig. 5.** mGX-sPLA<sub>2</sub> applied from IVM to IVF stages improves both embryo production and blastocyst hatching.

(A) mGX (200 nM) was applied from IVM to IVF steps. (B) Yield of 2-cell embryos on day 2 for gametes/embryos incubated in control and mGX-supplemented media. (C) Yield of blastocysts on day 7. (D) Yield of hatched blastocysts on day 9. n = 3 replicates. Range of oocytes number per condition: 65-75. Statistical differences were assessed using a paired t-test.

**Fig. 6.** Enzymatically-inactive H48Q-mGX mutant fails to enhance *in vitro* embryo production.

(A) The enzymatically-inactive H48Q-mGX mutant (200 nM) was applied from IVM to IVF steps. (B) Yield of 2-cell embryos on day 2 for gametes/embryos incubated in control and H48Q-mGX-containing media. (C) Yield of blastocysts on day 7. (D) Yield of hatched blastocysts on day 9. n = 6 replicates. Range of oocytes number per condition: 200-270. Statistical differences were assessed using a paired t-test.

**Fig. 7.** Bovine group IB (bGIB) sPLA<sub>2</sub> applied from IVM to IVF stages adversely impacts *in vitro* embryo production rates.

(A) bGIB (200 nM) was applied from IVM to IVF steps. (B) Yield of 2-cell embryos on day 2 for gametes/embryos incubated in control or bGIB-supplemented media. (C) Yield of

blastocysts on day 7. (D) Yield of hatched blastocysts on day 9. n = 3 replicates. Range of  
410 oocytes number per condition: 65-75. Statistical differences were assessed using a paired t-test.

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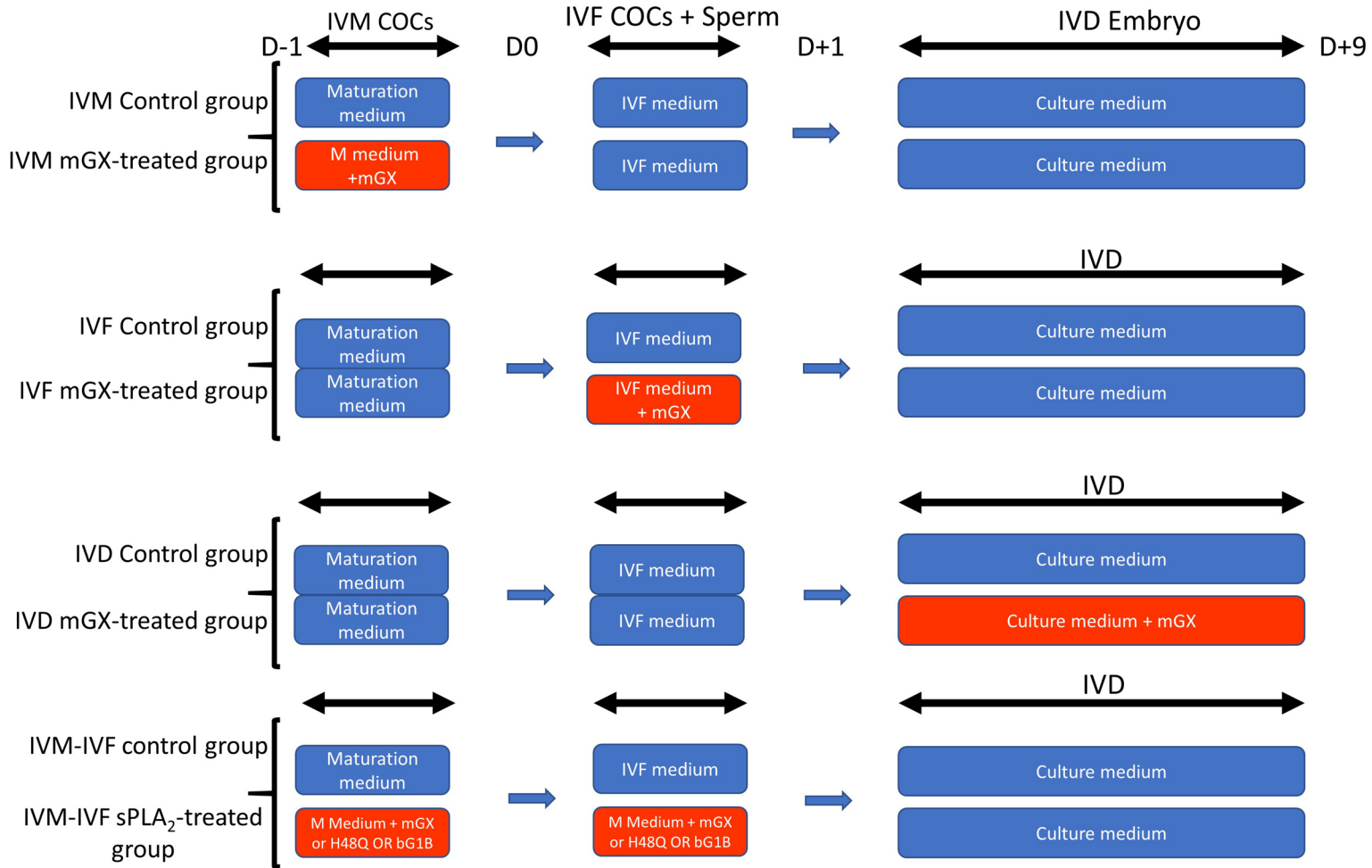
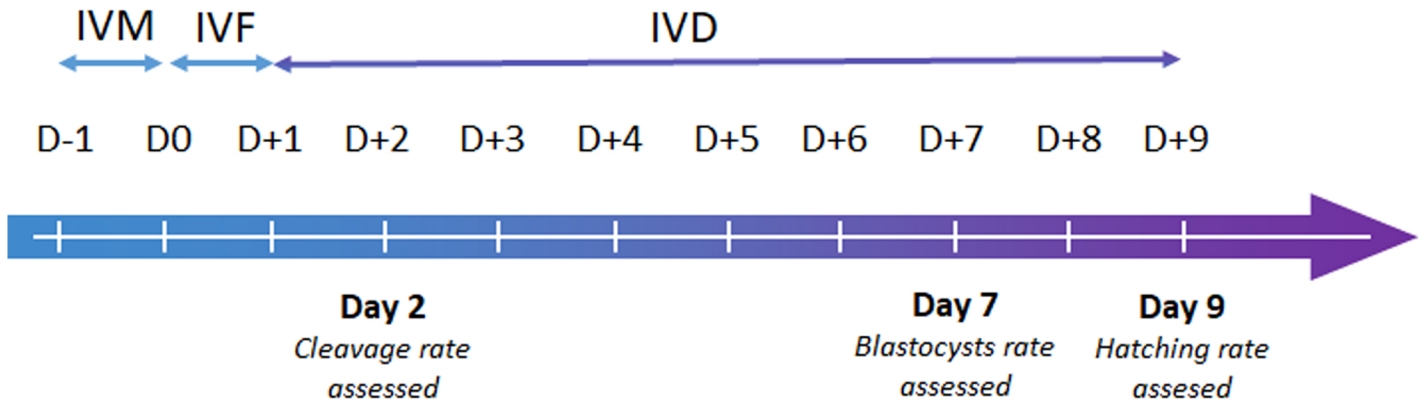
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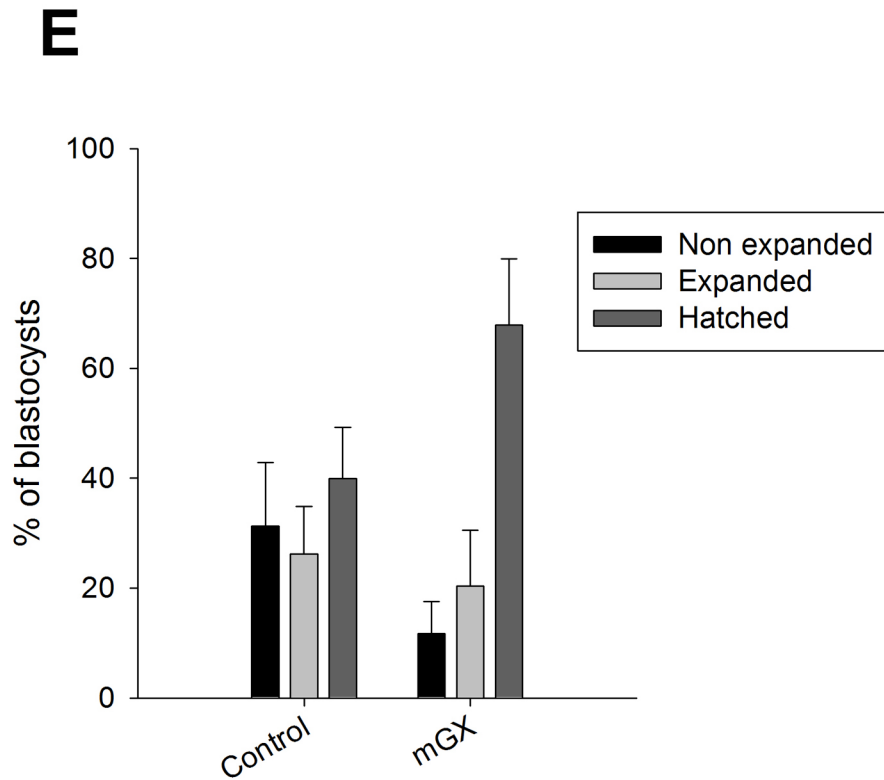
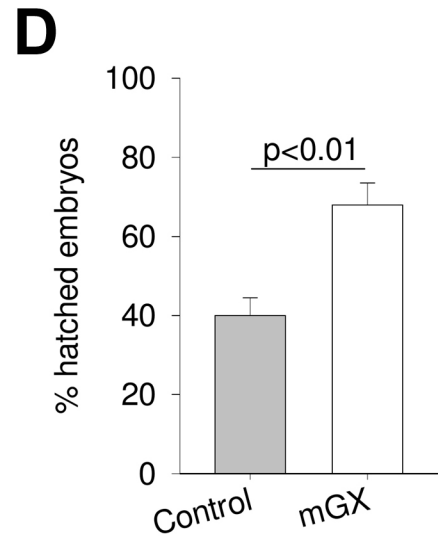
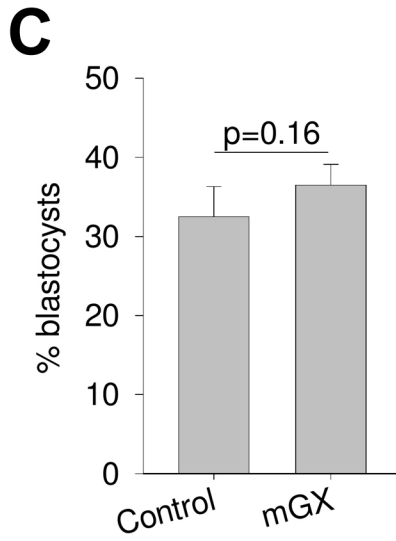
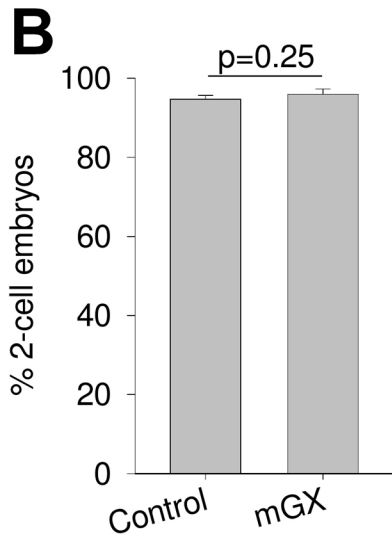
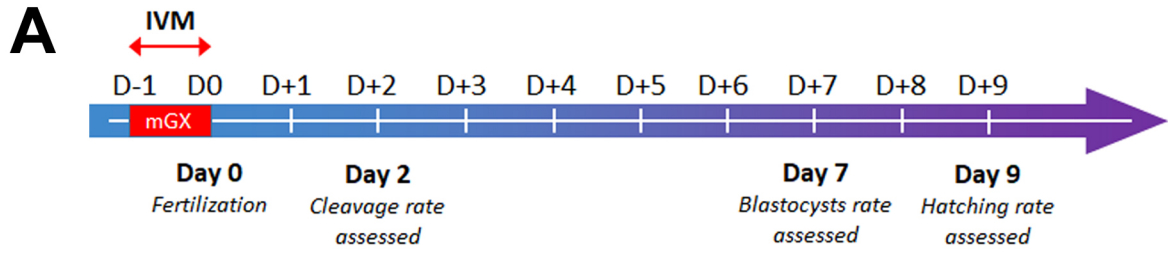
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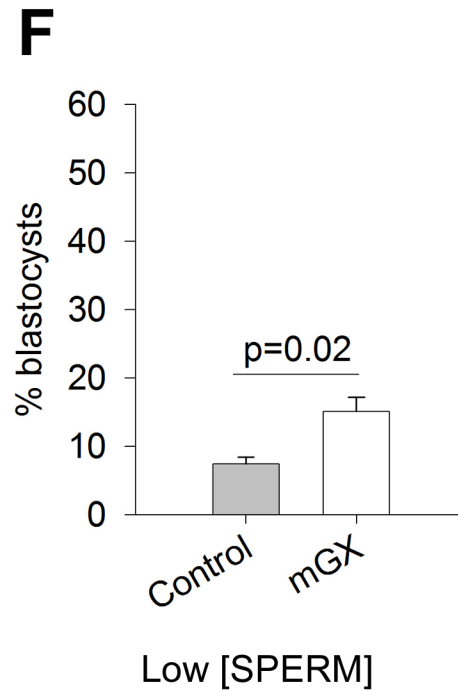
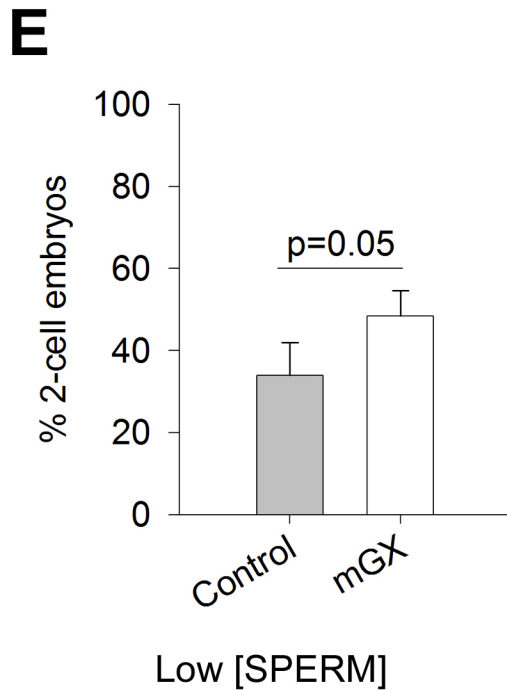
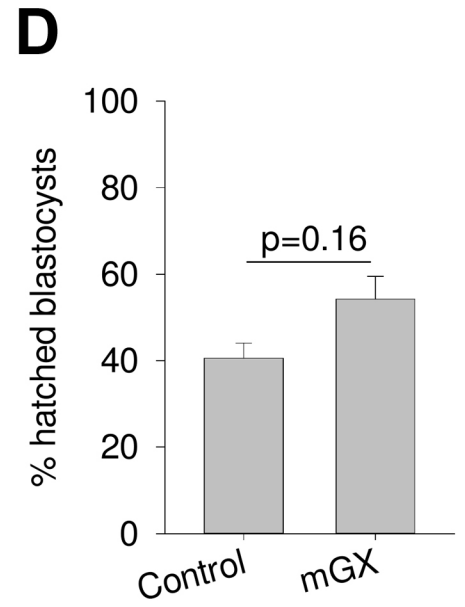
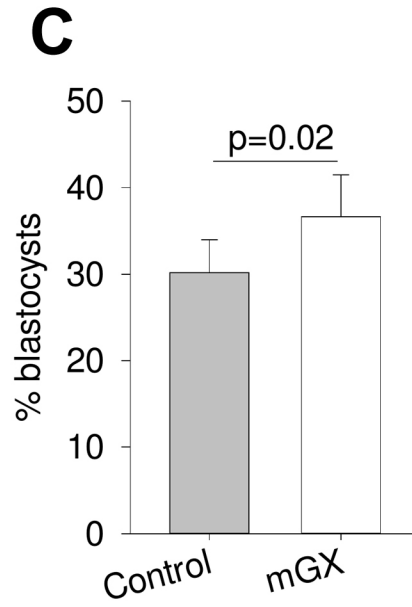
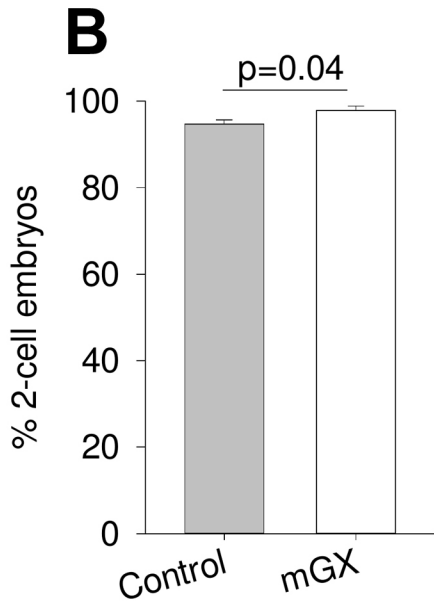
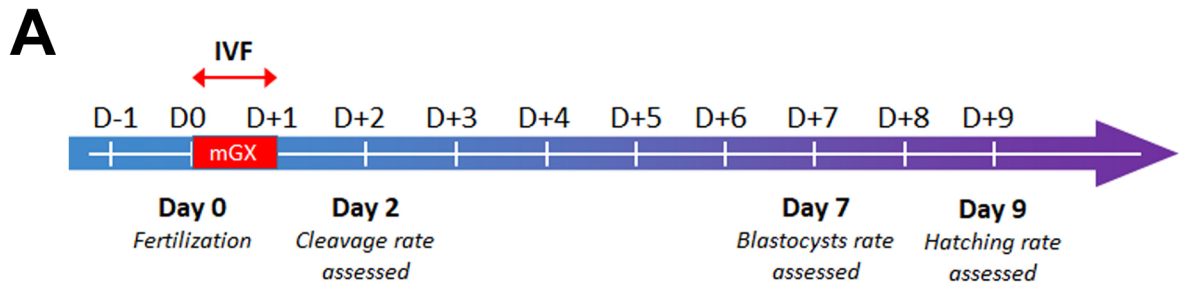
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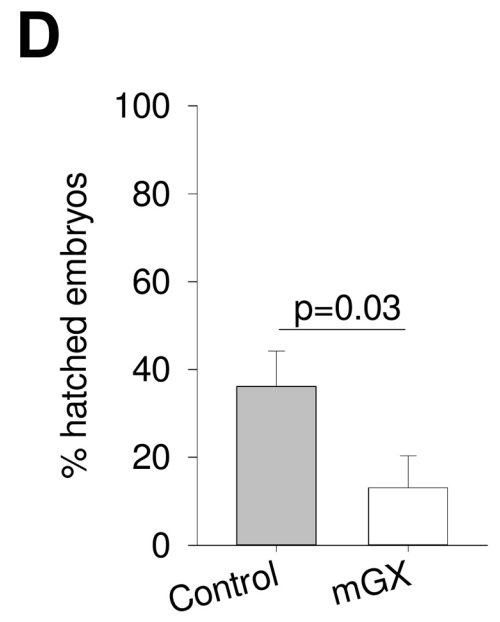
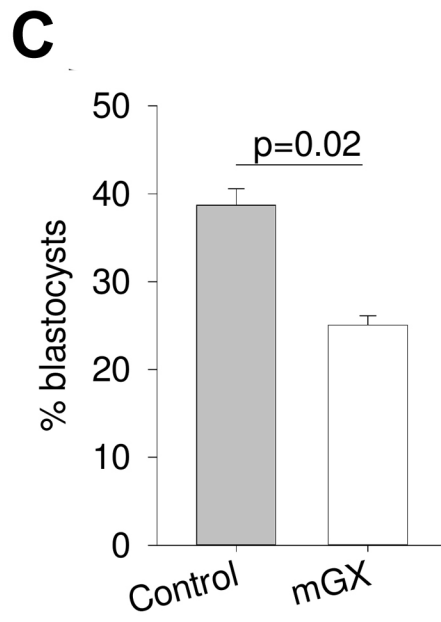
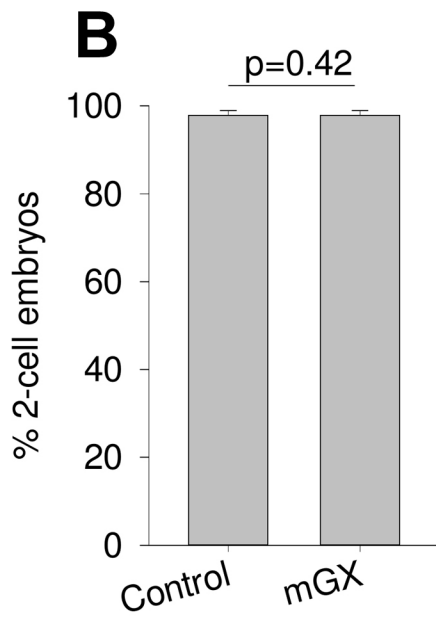
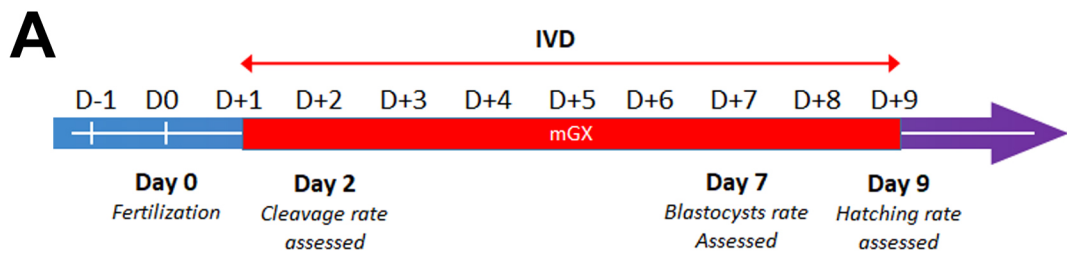
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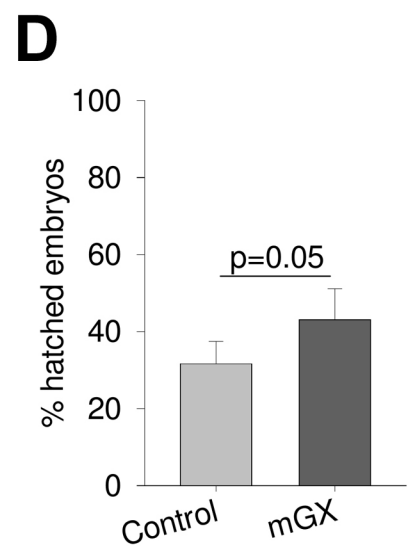
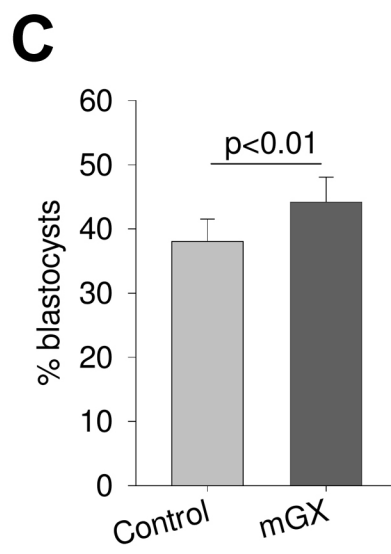
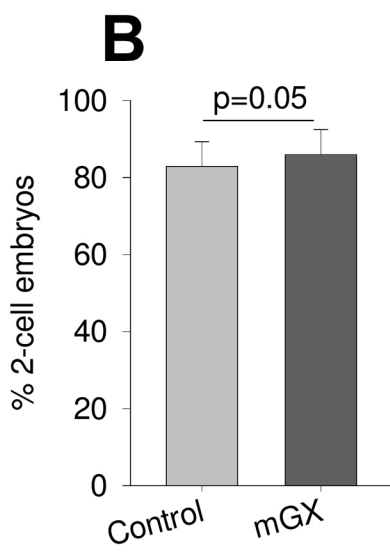
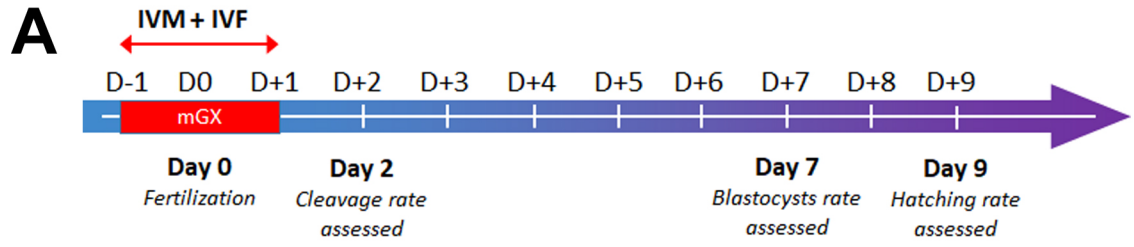


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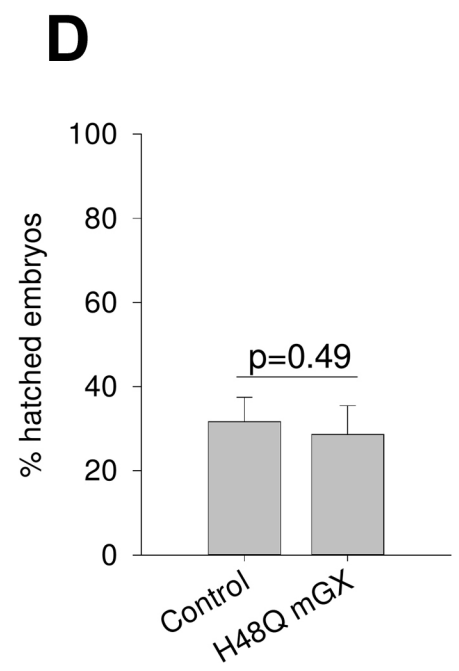
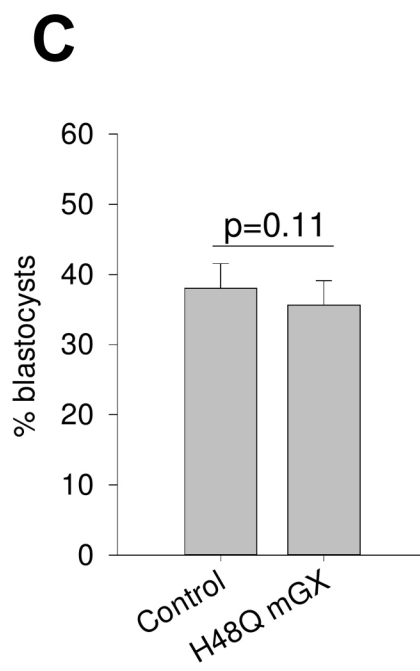
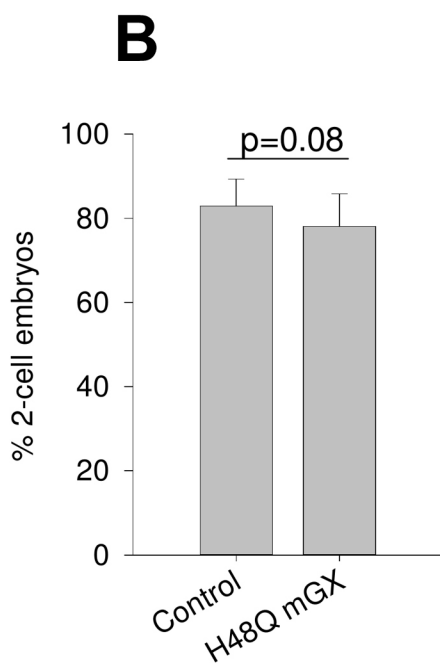
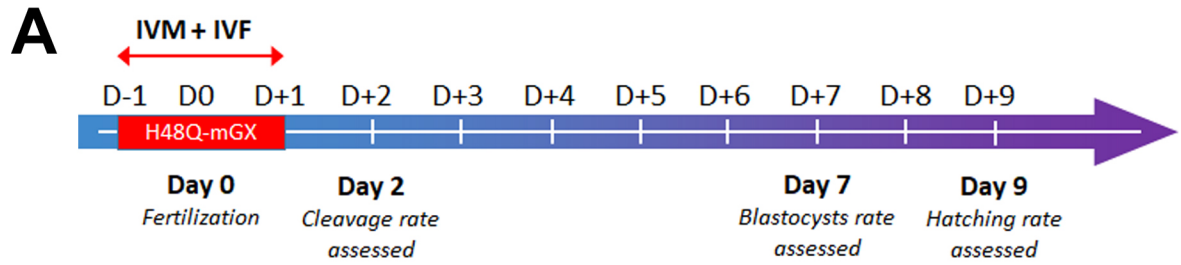




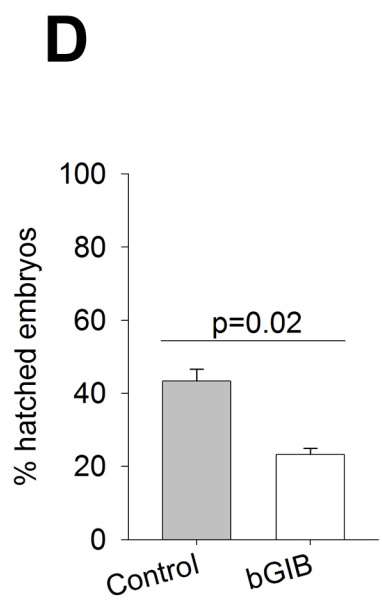
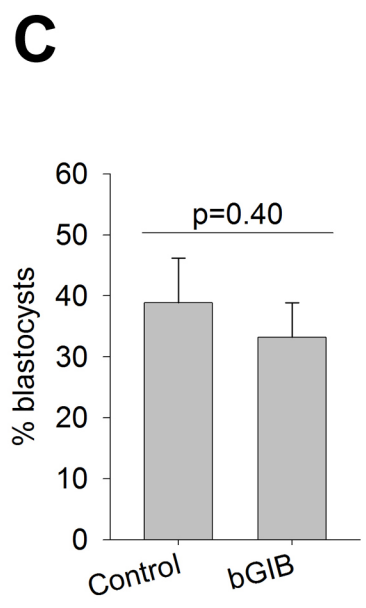
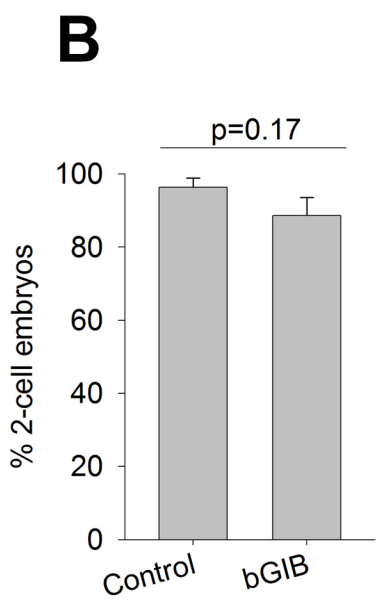
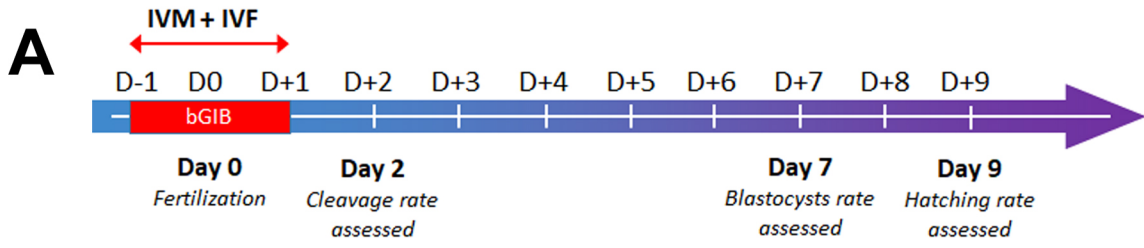
Martinez et al, Figure 4



Martinez et al, Figure 5



Martinez et al, Figure 6



Martinez et al, Figure 7