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Carbon Monoxide Exposure Does Not Improve The *In Vitro* Fertilization Rate of Oocytes Obtained from Heterozygous *Hmox1* Knockout Mice

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Abstract

In our experimental study we explored the impact of maternal reduced heme oxygenase-1 (HO-1) gene (*Hmox1*) expression on the *in vitro* fertilization (IVF) rate through the use of heterozygous *Hmox1* knockout mice models (*HET/Hmox1*$^{+/-}$). Also, we hypothesized a beneficial role of gametes exposure during fertilization to carbon monoxide (CO), one of HO-1 by-products, that might be relevant for the improvement of IVF rates. IVF technique was performed by using oocytes obtained from wild-type (WT) or *Hmox1*$^{+/-}$ dams fertilized with WT, *Hmox1*$^{+/-}$ or *Hmox1*$^{-/-}$ mice-derived sperm. The fertilization step occurred either in a conventional incubator (37°C, 5% CO_2) or in an incubator implemented with CO (500 ppm). The superovulation yield of WT and *Hmox1*$^{+/-}$ mice and the number of fertilized oocytes was assessed using an optical microscope. The dams' *Hmox1* heterozygous knockout neither impact the superovulation yield, nor did influence the fertilization success rate. Moreover, CO exposure during fertilization could not significantly improve the outcome. Our study showed that the maternal *Hmox1*$^{+/-}$ condition is not affecting the IVF rate in mice. Furthermore, we discovered that CO exposure cannot be exploited to ameliorate this critical step of the IVF protocol.

Keywords: Carbon Monoxide, Gene Knockout, HO-1 Protein, *In Vitro* Fertilization, Pregnancy

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Fertilization failure is still a major concern of the *in vitro* fertilization (IVF) procedure (1). In particular, total fertilization failure (TFF), which refers to the complete absence of fertilized oocytes after the incubation step with the donor sperm, occurs in 5-10% of IVF cycles (2). The intracytoplasmic sperm injection (ICSI) technique was introduced to overcome the possible obstacles related to the sperm ability to fuse with the egg. Nevertheless, TFF after ICSI occurs in the 2-3% of the cases, and generally up to 30% of the oocytes are not fertilized (3). This suggests that not only the paternal side, but the maternal as well is determinant for a successful fertilization, since sperm penetration is only one of the several events which need to occur efficiently. The age of the maternal donor is one of the main limitations to a successful outcome, however fertilization problems might occur even if the female donor is in the recommended age range to undergo IVF (< 38 years old), and when oocytes of at least apparent good quality and sperm of proven fertility are utilized (4). For this reason, it is crucial to gain a better understanding of possible genetic aspects and specific modulators that might be involved in the fertilization stages to support the choice of the right IVF approach. Besides, this additional knowledge could be relevant to optimize the current available methodologies.

Recent studies enlightened the involvement of heme oxygenase-1 (HO-1), the ubiquitous stress-induced isoform of HO, in diverse pregnancy-related processes, including fertilization (5, 6). HO-1 is encoded by the *HMOX1* gene, which was found to be expressed in both placenta and fetus starting from early gestational stage (7). HO is primarily known for catalysing the initial and rate-limiting reaction of heme degradation, and together with its by-products carbon monoxide (CO), biliverdin, and ferrous iron (Fe^{2+}), exhibit important cytoprotective, immunomodulatory, antioxidant, and anti-inflammatory properties (8). Remarkably, HO-1 deficiency in mice is correlated to diverse pregnancy complications, such as growth restriction,

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fetal loss, defective spiral artery remodeling, and hypertension. It was postulated that excess of free heme and insufficient release of CO are the main causes for this outcome. Indeed, application of hemin (free heme) dramatically increased fetal death (9). Further, the administration of low doses of CO (50 ppm) to HO-1 partial and total deficient dams at the time of placenta development (days 3-8 of pregnancy) was able to promote fetal viability, increase both placenta and pups' weights, and ameliorate the systolic blood pressure and wall-to-lumen ratios of the spiral arteries (9, 10). Accordingly, a possible role of the HO/CO axis in ovulation and fertilization was also investigated. It was previously shown that total-deficient (KO/*Hmox1*$^{-/-}$) female mice had a less efficient ovulation, and that the oocytes harvested from these dams were significantly less fertilized compared to those obtained from wild-type (WT) ones (20 vs. 60%) (6). Considering that sperm from WT and *Hmox1* knockout (KO) mice were proven to be equally fertile (11), these evidences proved that the total lack of HO-1 in the oocytes influences their ability to successfully complete fertilization, which includes several post-sperm penetration events. However, HO-1 human deficiency is a rare condition and up to date only 9 cases were described of patients who exhibited devastating and complex clinical courses (12). Nonetheless, it was described that *HMOX1* expression in the population may vary due to short tandem GT_n repeat (STR) region present in the promoter (13, 14). In particular, longer repeats were associated to decreased *HMOX1* expression in response to oxidative stress and to reduced HO-1 levels in serum and placenta (15-17).

In line with the described considerations and based on previous findings, the aim of the present study was to investigated whether a reduced maternal *Hmox1* expression in mice has an impact on ovulation and on the IVF success using heterozygous *Hmox1* knockout models (HET/*Hmox1*$^{-/-}$). Furthermore, we aimed to explore whether the exposure of oocytes to CO is able to improve their fertilization rate.

The progeny obtained by mating *Hmox1*$^{+/-}$ females with *Hmox1*$^{+/-}$ males in a BALB/c background was genotyped and accordingly assigned to the *Hmox1*-wild type (WT/*Hmox1*$^{+/+}$), *Hmox1* heterozygous KO (HET/*Hmox1*$^{+/-}$), or *Hmox1* total-KO (KO/*Hmox1*$^{-/-}$) mice groups that were used for the experiments (9). Mice were maintained in our barrier facility with a 12-h light/dark cycle, 22 ± 2°C, and 40-60% air humidity. They received water and food *ad libitum*. Experimental procedures were performed according to the institutional guidelines and approved by German authorities (Landesverwaltungsamt Sachsen Anhalt: 42502-2-1327 Uni MD).

The IVF protocol used in our experimental study included the following steps. Firstly, superovulation of WT (n=21) or *Hmox1*$^{+/-}$ (n=22) dams was induced by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG, Integonan®, Intervet, Germany), followed by 5 IU of Ovogest® (human chorionic gonadotropin, Intervet International B.V., Kenilworth, NJ, USA) 48 hours later. 12 hours later, sperm was collected from the cauda epididymis and the vasa deferentia of the males and transferred to a 35 mm petri dish containing 1 ml of ORIGIO® Handling™ IVF Medium (CooperSurgical®, Målov, Denmark), where it was incubated for 10 minutes at 37°C. Afterwards, 10 µl of the sperm were incubated (37°C, 5% CO_2) for 1 hour within 500 µl of pre-warmed IVF medium in 80 mm petri dishes ("IVF dishes") coated with mineral oil (Sigma Alderich, Steinheim, Germany), which were prepared the day before and incubated overnight (37°C, 5% CO_2). At this point-approximately 14 hours after Ovogest® injection-the dams were sacrificed by cervical dislocation. The oocyte complexes were collected from the ampullae and transferred to 35 mm petri dishes containing 1 ml of pre-warmed of IVF medium each. Oocytes that were obtained from each female were counted.

For the IVF experiments, we selected females from which we could harvest a minimum number of 5 oocytes. These females were randomly divided into 2 groups (control or CO-treated). This resulted in the following subgroups: WT-control (n=7), WT-CO-treated (n=7), *Hmox1*$^{+/-}$ -control (n=6), *Hmox1*$^{+/-}$ -CO-treated (n=7). Right after, the oocyte complexes were added to the IVF dishes in the same drop which contained the capacitated sperm. The sperm of one male was used to fertilize 4 females, each to cover all groups (WT-control, WT-CO-treated, *Hmox1*$^{+/-}$ -control, *Hmox1*$^{+/-}$ -CO-treated). The oocytes were then incubated for 4-6 hours at 37°C in a classic CO_2 incubator or in one implemented with CO at a concentration of 500 ppm, which was previously successfully used in one *in vitro* study (9). After, the oocytes were washed to remove the excess of sperm using a capillary to aspirate and move them in four 150 µl drops of IVF medium present in the IVF dishes. The washed oocytes were further incubated overnight at 37°C in the CO_2 incubator with or without CO (500 ppm). The morning after the number of fertilized ones was assessed via optical microscope observation. The fertilization rate was then calculated as the ratio of fertilized oocytes (two-cells stage embryos) divided by the total number of oocytes, multiplied by 100 (Fig.1).

GraphPad Prism software version 8.0 (GraphPad, Statcon, Witzenhausen, Germany) was used for the statistical analysis. For all the data collected mean ± SEM was calculated. In particular, normal distribution of the data using Shapiro-Wilk test was assessed. An unpaired t test was performed to establish the statistical difference in the number of oocytes harvested from the two groups of dams, while the data obtained from the fertilization experiments were analysed with Brown-Forsythe and Welch ANOVA test. All the groups' means were compared to each other using Dunnett's T3 test for multiple comparison as post-hoc test.

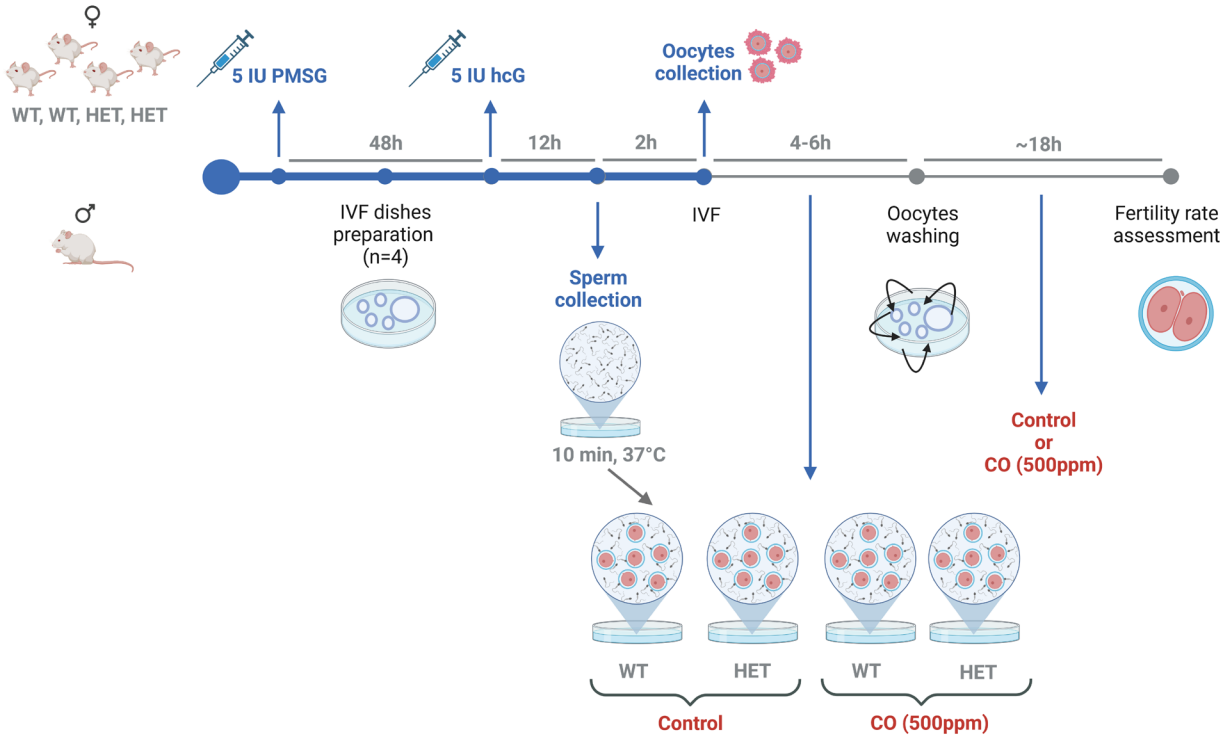

Fig.1: Graphical representation of the IVF procedure and experimental design. One experimental block comprised three rounds of IVF and was repeated three times in total. In each round the sperm from one male was used to fecondate the oocytes retrieved from 4 females (two WT and two HET dams) after the induction of superovulation by the intraperitoneal injection of 5 IU of PMSG, followed by 5 IU of Ovogest® . From the starting number of dams available, only those from which a minimum number of 5 oocytes could be harvested were used and randomly assigned to the control or CO-treated group. This resulted in the following dams number per subgroup: WT-control (n=7), WT-CO-treated (n=7), *Hmox*$^{+}$/$^{-}$-control (n=6), *Hmox*$^{+}$/$^{-}$-CO-treated (n=7). Their oocytes were correspondingly incubated with the capacitated sperm for 4-6 hours at 37°C either in a classic CO_2 incubator or in one implemented with CO at a concentration of 500 ppm. The oocytes were then washed to remove the excess of sperm and further incubated overnight at 37°C in the CO_2 incubator with or without CO (500 ppm). The morning after, the fertilization rate was then calculated as the ratio of fertilized oocytes (two-cells stage embryos) divided by the total number of oocytes, multiplied by 100. Created with BioRender.com. IVF; *In vitro* fertilization, WT; Wild type, HET; Heterozygous knockout for *Hmox1* (*Hmox*$^{+}$/$^{-}$), PMSG; Pregnant mare serum gonadotropin, CO; Carbone monoxide, h; Hours, and hCG; Human chorionic gonadotropin.

To begin with, we evaluated if the decreased dams *Hmox1* expression could have an impact on their ovulation. The mean of the oocytes obtained after the superovulation was comparable between the WT and *Hmox1*$^{+}$/$^{-}$ groups (Fig.2). This indicates that the ovulation process in *Hmox1*$^{+}$/$^{-}$ females is not impaired as it is in full KO mice (6). The oocytes retrieved (n=6-26) from WT or *Hmox1*$^{+}$/$^{-}$ mice were incubated with the capacitated sperm either in a classic incubator (5% CO_2, 37°C) or in an incubator implemented at a CO concentration of 500 ppm (9). To exclude falsification of the results due to differences in sperm fertility, the sperm of one male was used to fertilize 4 females, each to cover all groups (WT-control, WT-CO, *Hmox1*$^{+}$/$^{-}$-control, *Hmox1*$^{+}$/$^{-}$-CO). A total number of 3 males per genotype was used as seed donors. Besides WT and *Hmox1*$^{+}$/$^{-}$, also *Hmox1*$^{-}$/$^{-}$ males were available and used for the experiments. Indeed, it was showed before that sperm from *Hmox1* WT and KO mice is equally fertile (11), and this was confirmed in our experiments as well (data not showed). Moreover, it was found that HO-1 is only moderately expressed in male germ cells, while the prevalent HO isoform is the constitutively expressed HO-2, which is abundantly present in the testis (18).

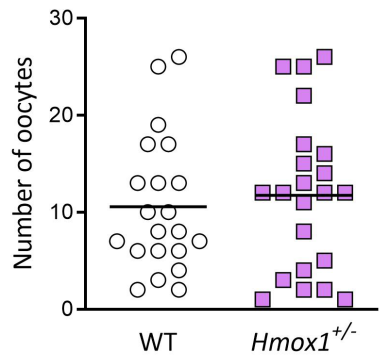

Fig.2: Number of oocytes harvested after superovulation from each wild-type (n=21) or *Hmox1*$^{+}$/$^{-}$ (n=22) dams. Data are shown as individual values with mean and were analysed for statistical differences by using the unpaired t test. WT; *Hmox1* wild type and *Hmox1*$^{+}$/$^{-}$; Heterozygous knockout for *Hmox1*.

As a result, the IVF experiments showed that the HO-1 partial deficiency of female mice did not lead to any statistically significant differences in the ability of their oocytes to be fertilized (Fig.3). This suggests that one maternal functional copy of the gene is enough to guarantee ovulation and fertilization at rates comparable with mice equipped with both copies.

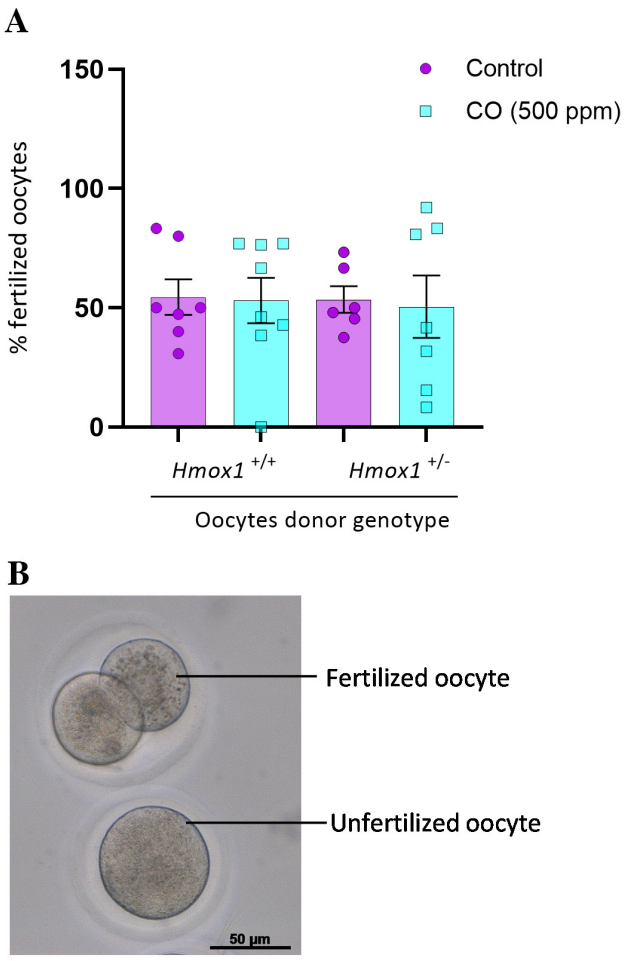

Fig.3: Percentage of fertilized WT or *Hmox*$^{+/-}$ dams- derived oocytes after CO treatment. **A.** Percentage of wild type (control n=7, CO-treated n=7) and *Hmox*$^{+/-}$ (control n=6, CO-treated n=7) dams-derived oocytes incubated in a CO_2 incubator without (control) or with CO (500 ppm). **B.** Microscopic image showing a fertilized (2-cells stage) and an unfertilized oocyte (scale bar: 50 µm). Data are presented as box plots showing the mean ± SEM and dots representing the individual values. The analysis for statistical differences was conducted using the Brown-Forsythe and Welch ANOVA test. Dunnett's T3 was used as post-hoc test. WT; *Hmox1* wild type and *Hmox*$^{+/-}$; Heterozygous knockout for *Hmox1*.

CO was demonstrated to mediate the protective functions of HO-1 enzyme in several pregnancy stages (5). Specifically, exogenous CO application was able to promote a normal placentation and fetal growth in mice which were totally defective for *Hmox1* expression and characterized by several pregnancy complications (9, 10). Additionally, the application of gaseous CO between gestation day 3 and 8 of pregnancy increases the percentage of viable fetuses in *Hmox1*$^{+/-}$ females mated with *Hmox1*$^{+/-}$ males (9). Dickson et al. (19) also demonstrated that pregnant dams exposure to CO (250 ppm) had a pro-angiogenic effect, leading to the enhancement of midgestational utero-placental vascular growth, with no negative impact on pregnancy outcomes. However, the effect of CO on oocytes or fertilization has been scarcely investigated. Interestingly, one study on *in vitro* oocytes aging showed that the delivery of CO to porcine oocytes was able to improve their viability and decrease the apoptotic rate mainly through the downregulation of caspase-3 activity. In the mentioned work different concentrations of CO donors (5-100 µM) with fast or slow release rates were used to implement the oocytes culture medium in presence of a heme oxygenase inhibitor (20).

In the presente study we wanted to explore whether CO exposure could improve the fertilization outcome in vitro. CO exposure during fertilization failed to improve the success rate of both WT and *Hmox1*$^{+/-}$ derived oocytes, since no statistically significant differences was observed between the groups' means (Fig.2). For future studies, different CO concentrations or alternative forms of exposure might be used to see if any remarkable changes are detected. A different question might be whether the formerly discovered low fertilization rate of oocytes derived from KO females would rather be improved by a treatment with CO. Unfortunately, the availability of female KO mice is very low. We previously found that 10 *Hmox1*$^{+/-}$×*Hmox1*$^{+/-}$ breeding pairs resulted in only 7 KO females within 1 year. In contrast to the expected 25%, only 8.46% of the pups exhibits a KO genotype, of which 5.68% males and 3.13% females (21). This low yield made logistically and technically impossible to answer the very interesting question formulated before.

For the IVF experiments, we used females from which we could harvest a minimum number of 5 oocytes.

For this reason, our initial mice cohort was reduced since we had to exclude few dams which produced a number of oocytes ranging from 1 to 4. Following this initial selection, in each single fertilization experiment the starting number of oocytes used ranged from 6 to 26. Still, we are aware that the sample size and the variation in the number of oocytes may have an influence on the calculated fertility success rate, and they might be considered limitations of our study. Also, it would be interesting to perform the same analysis taking into consideration the genotype of the embryos, to investigate whether the embryonic *Hmox1* expression at the very early beginning of pregnancy is crucial to terminate the whole fertilization process successfully and further steps of the IVF protocols. These aspects were not considered in the present study and should be taken into account in the future.

In conclusion, we demonstrated that the maternal *Hmox1*-heterozygous KO condition in mice did not affect the ability of their oocytes to be fertilized *in vitro*. Also, contradicting the initial hypothesis of therapeutic use, we showed that CO treatment did not affect the IVF rate, at least of WT or *Hmox1*$^{+/-}$ female derived oocytes. Nevertheless, it is conceivable that CO can be used for therapeutic purposes to compensate for various pathological consequences of a disturbed pregnancy, possibly at a later time in the course of a natural or IVF pregnancy.

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Authors' Contributions

F.R.; Performed writing (original draft, review and editing), Validation, Formal analysis, and Data visualization. N.M.; Contributed with conceptualization, Project administration, Investigation, Review and editing. M.L.Z.; Participated in the review and editing process. A.C.Z.; Contributed with methodology, Supervision, Funding acquisition, Review and editing. All authors read and approved the final manuscript.

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