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Preconceptional paternal glycidamide exposure affects embryonic gene expression: Single embryo gene expression study following *in vitro* fertilization

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ABSTRACT

Recognition of early determinants of disease onset has sparked an interest in paternally transmitted factors and their impact on the developing embryo. Acrylamide (AA), a widely distributed xenobiotic compound, is converted to its active metabolite glycidamide (GA) by the CYP2E1 enzyme. Based on its capacity to induce dominant lethal mutations, we hypothesized that paternal GA exposure would have a negative impact on embryonic genome activation, via GA-DNA and protamine adducts persisting in the fertilizing sperm. Using a combination of *in vitro* fertilization (IVF) techniques and RT-qPCR single embryo gene expression (SEGE), we studied the expression of key DNA repair genes and genes important for embryo development, at the 1-, 2-, 4- and 8-cell stage of the developing mouse embryo. Compared to controls paternal GA-exposure gave rise to an altered pattern of embryonic gene expression, with an initial reduced expression at early stages followed by increased expression at the 8-cell stage.

1. Introduction

A considerable part of infertility problems among couples is believed to be caused by male factors [1], and there is evidence of a decline in semen quality in many industrialized countries [2,3]. A parallel increase in testicular cancer observed over the last decades [4–6] raises concern over the impact of a modern lifestyle on the male reproductive system. Environmental factors are believed to be implicated in both the diminishing male reproductive health and the increased cancer incidence, but the part of the modern environment posing the greatest threat towards the male reproductive system has not been determined.

Germ line gene–environment interactions are currently being explored in an attempt to understand the dissonance between male reproductive health and the modern environment. Concern has been expressed on the possibility that paternal DNA damage is propagated across multiple generations. Post-meiotic male germ cells are sensitive to induction of heritable genomic damage particularly during the last few weeks of spermatogenesis [7,8]. All major DNA repair pathways seem to be less functional in late spermatids and sperm [9–12]. We have previously reported on excision repair activities in different testicular cell types [10,13–15] confirming that DNA lesions are not repaired in late spermatids and sperm. High levels of DNA lesions are thus present in the sperm [15–17]. Embryonic development may be compromised when oocytes are fertilized with sperm containing higher than normal levels of DNA- and protamine-adducts.

Acrylamide (AA) is one of many chemicals that have been shown to induce DNA damage in the male germ line [18–20], dominant lethal mutations representing one critical consequence [20,21]. In addition to being an industrial chemical and a component of coffee and cigarette smoke, AA is formed by high temperature processing of glucose and, it is generally present at low levels in many carbohydrate rich fried foods like French fries, potato crisps, crisp bread, bread, and biscuits [22–24]. AA is oxidized to the reactive electrophilic epoxide glycidamide (GA) [25] by the CYP2E1 enzyme [26–28]. A large part of the damages attributed to AA exposure is believed to be caused by this reactive metabolite [26,28–30]. DNA and protamine alkylation have been suggested as mechanisms by which AA induces germ cell mutagenic effects [19,31,32], the most commonly reported DNA adducts being N7(2-carbamoyl-2-hydroxyethyl)-guanine (N7-GA-Gua) and to a lesser extent N3(2-carbamyl-2-hydroxyethyl)-adenine (N3-GA-Ade) [26,33–35].

Global activation of the embryonic genome constitutes the most critical event at early stages of mammalian development. Maternal proteins and RNAs support development after fertilization, whereas a number of zygotic and embryonic genes are expressed

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in a stage specific manner leading to embryonic genome activation [36]. The maternal to zygotic transition can be subdivided into two interrelated processes; first, a subset of the maternal mRNAs and proteins is eliminated; second, zygotic transcription is initiated [37]. The maternal to zygotic transition is a highly coordinated and extremely complex biochemical symphony and it has been estimated in the mouse that about 15,700 genes are expressed during preimplantation development [38]. Paternal exposure to the anticancer alkylating agent cyclophosphamide has been shown to alter the expression of DNA repair genes in the rat pre-implantation embryo [39,40]. A relevant question is whether less potent and more widely distributed environmental chemicals can affect the developing embryo via the same exposure route.

Based on our previous findings of very high levels of DNA lesions in sperm [15,16] and the ability of GA to form DNA and protamine adducts, we hypothesized that paternal preconceptional exposure to GA would have an impact on early embryonic transcription and on the activation of the embryonic genome. We studied the expression of key DNA repair genes and genes important to embryo development following paternal acute exposure to GA seven days prior to fertilization. To our knowledge this is the first time RT-qPCR single zygote/embryo gene expression techniques are used in studies on reproductive toxicity. We demonstrate that the early embryonic transcription of multiple genes is affected after paternal germ cells exposure to GA. The RT-qPCR single embryo gene expression technique has potential as a tool to study early developmental toxicity.

2. Methods

2.1. The exposure of male mice from which sperm was derived for IVF

Exposed males (strain B6D2F1 from Charles River Laboratories, 8–12 weeks of age) received one i.p. injection of GA (61 mg/kg body weight) dissolved in phosphate buffered saline eight days prior to the IVF experiment. Timing of the exposure to GA was based on pilot studies and knowledge about the most susceptible stage of spermatogenesis with respect to dominant lethal mutations. Similarly aged control males received an equivalent volume of phosphate buffered saline. At the day of the IVF experiment males were killed by cervical dislocation. Cauda were surgically removed and collected in an eppendorf tube containing M2 medium (500 μl, Sigma). Using a pair of micro scissors a few incisions were made in the cauda and the sperm was allowed to disperse for 10 min in a small drop (250 μl) HTF medium (EmbryoMax, Millipore) under liquid paraffin (MediCult) before transfer to the IVF dishes. Experiments are based on oocytes from 75 females and sperm from 10 males (5 exposed and 5 controls) altogether.

2.2. Super-ovulation and in vitro fertilization (IVF)

Females (strain B6D2F1 from Charles River Laboratories, 4–6 weeks of age) were injected i.p. with pregnant mare serum hormone gonadotropine (PMSG, Folligon from Intervet) (5 IU) three days prior to the IVF procedure. Two days later (that is, the day before the IVF) animals received an additional i.p. injection of human chorionic gonadotropine (HCG, Ovitrelle from Serono) (5 IU). Mice were killed by cervical dislocation and oviducts were collected in M2 medium (Sigma). Egg clutches (10–20 oocytes) embedded in cumulus cells were extracted from each oviduct. Oocytes were transferred to IVF-dishes and incubated in a droplet of HTF sperm containing medium under liquid paraffin for 4.5 h (37 °C). Oocytes from one side of the animal were combined with sperm from gycidamide exposed animals and oocytes from the other side were combined with sperm from control animals. Hence, oocytes from all animals were present in both the control group and the exposed group. After 4.5 h the fertilized oocytes (zygotes) were washed 5× in KSOM medium (EmbryoMax Millipore) before they were transferred to a drop of KSOM (200 μl) in a petri dish (35 mm) under liquid paraffin (MediCult). Samples from the 1-cell stage were collected immediately after fertilization. The rest of the zygotes were allowed to grow to harvest at the 2-cell, 4-cell or 8-cell stage. Upon harvest zygotes/embryos were collected in micro tubes filled with 5 μl lysis medium (CelluLyser, TATAA) and then frozen at −70 °C.

2.3. Reverse transcriptase and cDNA synthesis

Samples where thawed before a total of 14.5 μl mastermix and reverse transcriptase enzyme (Roche transcriptor first strand cDNA synthesis kit, Cat no. 04896866001) was added to all samples. The samples were then incubated in a thermal cycler unit (Eppendorf mastercycler) according to the following protocol: 10 min at 25 °C, 30 min at 50 °C, 5 min at 85 °C, and then held at 4 °C. After the RT reaction was completed samples were again frozen at −70 °C.

2.4. Preamplification of cDNA

Preamplification was used to increase the number of template molecules. This is a necessary step because the cDNA synthesis does not yield sufficient number of molecular copies of the template molecules which can be analyzed with confidence in parallel singleplex reactions. A high similarity between the gene expression measurements of preamplified and non-preamplified samples has been reported [41]. Preamplification PCR was run in 20 μl volume containing 4 μl of cDNA, 2 μl of a mixture of all forward and reverse primers (500 nM each), 10 μl of AmpliTaq Gold360 Master mix (Applied Biosystems) and water. PCR primers were designed using the online Universal Probe Library System from Roche. A CFX 96 cycler (Bio-Rad) was used for the preamplification with the cycling conditions: polymerase activation at 95 °C for 10 min, followed by 18 cycles (95 °C 15 s, 59 °C 1 min and 72 °C 1 min). The product of the preamplification reaction was diluted to 80 μl and stored at −20 °C. The robustness of the preamplification was validated by comparing qPCR expression levels of Chek (highly expressed) and Mlh3 (typically low expression) with and without preamplification in tissues from liver, ovaria and embryo. The relative expression of the two genes was similar when analyzing data with and without preamplification.

2.5. High throughput qPCR

The sample reaction mixture had a volume of 5 μl and contained 1 μl of preamplified cDNA, 0.5 μl of SYBR Green Sample Loading reagent (Fluidigm), 2.77 μl AmpliTaq Gold360 Master mix (Applied Biosystems), 0.165 μl of Chromofy, diluted 1:25 (TATAA), 0.025 μl of ROX (Invitrogen). The primer reaction mixture had a final volume of 5 μl and contained 2.5 μl Assay Loading Reagent (Fluidigm) and 2.5 μl mixture of reverse and forward primers corresponding to a final concentration of 5 μM. The chip was first primed with an oil solution in the NanoFlex™ 4-IFC Controller (Fluidigm) to fill control wells of the dynamic array. Bubbles were carefully removed from the 5 μl reaction mixture and the mix was loaded into the sample wells, and 5 μl of the primer reaction mixtures was loaded into the assay wells of the dynamic array. The dynamic array was then placed in the NanoFlex™ 4-IFC Controller for automatic loading and mixing. After 55 min the dynamic array was transformed to the BioMark qPCR platform (Fludigm). The cycling program was 10 min at 95 °C for preactivation, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 20 s. After completed qPCR cycling melting curves were collected between 60 and 95 °C with 0.5 °C increments.

2.6. qPCR basic data analysis

The BioMark qPCR platform is primarily designed for Taqman probes. Here we used next-generation Chromofy as an unspecific dye binding double stranded DNA with higher affinity and fluorescence compared to regular SYBR Green. Chromofy has excitation/emission spectra similar to FAM. Since there is no separate set up for Chromofy in the Biomark qPCR platform, we used FAM-MGB settings with slightly altered exposition temperature and a linear baseline correction derived from independent experiments (data not shown). ROX (emission at 645 nm) was always used for passive signal normalization.

2.7. Data preprocessing

Normalization of qPCR data is often done relative to the expression of reference genes, number of cells, weight of tissue (DNA/RNA spike) or total RNA concentration. Expression of mouse reference genes in temporal development of an embryo will not be constant and hence will likely introduce unwanted variability. As a consequence we chose to not to use endogenous reference genes in the present study, in line with recommendations from Sindelka et al. [42]. By not using endogenous reference genes emphasis was put on careful and highly standardized technical handling.

To ensure that measurements at low levels were well within the linear area of detection, all *Cq* values above 26 were coded as missing values. In addition, embryos with gene expression pattern radically different from the overall group mean were classified as outliers. The excluded outliers most likely represented deteriorating embryos that were about to die. Filtering criteria for missing values was set to 70%, which is the minimum percentage of existing values, and all the patterns with less than 70% existing values were removed. All estimates are mean values based on 10–15 single zygotes/embryos.

The information contained in the biological replicates was used to replace missing values in the remaining biological replicates when available. If all biological replicates gave missing data, they were all assigned the highest measured *Cq* of that particular gene +1. Since the highest measured *Cq* of a truly positive sample can be assumed to be the limit of detection (LOD) for that particular gene, assigning *Cq*(LOD) + 1 to the off-scale samples corresponds to a concentration that is half of the LOD. Relative expression (RQ) among the sample was calculated as [41]:

$$RQ = 2^{Cq_{min} - Cq}$$