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# The Addition of Antibiotics to Embryo Culture Media Caused Altered Expression of Genes in Pathways Governing DNA Integrity In Mouse Blastocysts

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Research Article

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## ABSTRACT

Antibiotics are common components of embryo culture media and minimize the risk of microbial contamination and infection during Assisted Reproductive Technology procedures (ART). The effects of two aminoglycoside antibiotics (gentamicin, streptomycin) and penicillin on the global profiles of Gene Expression (GE) were assessed by RNA-seq of individual mouse blastocysts. Zygotes were cultured in an optimized defined medium formulation (KSOM) to which a dose range of each antibiotic was added. A dose-dependent retardation of the rate of zygote development to morphologically normal blastocyst was observed and this was accompanied by a reduction in the number of cells present within the resulting blastocysts. These blastocysts exhibited the lower ability in further 96 hours outgrowth *in vitro*. The lowest dose of each antibiotic tested (similar to the concentrations used in clinical grade media) caused significant differential expression of approximately 1800 genes. In most cases antibiotic treatment caused a reduction in gene expression and gene ontology analysis showed that down regulated genes were enriched for several biological processes related to the maintenance of genomic integrity. All three antibiotics caused the downregulation of Brca2, Blm, Rad51c and Rad54l, genes involved DNA homologous recombination pathways and also several $p^{53}$-dependent genes. Immunolocalization studies showed that each antibiotic also reduced level of BRCA2 and RAD51C detected within blastocysts. The present study shows that the supplementing embryo culture media with antibiotics is associated with wide ranging alterations in gene expression in a manner that could potentially compromise the genomic integrity of the resulting embryos.

**Keywords:** Antibiotic; Differential gene expression; DNA recombination; Embryo development; Gene otology; *In vitro* fertilization; Signal pathway; Tumorigenic

## INTRODUCTION

The creation of embryos by *in vitro* fertilization and embryonic culture procedures is an important treatment of infertility in humans. Is generally recognized that the resulting embryos have a lower viability and developmental potential than embryos conceived naturally. There have been many developments in embryo culture techniques, including improved media formulations, yet the addition of antibiotics remains a common component of these procedures and serves as a quality assurance against microbial infection of cultures *in vitro* [1,2]. The types of antibiotics used and their concentration vary between media formulations. The most commonly used antibiotics are penicillin, streptomycin or gentamicin.

The aminoglycoside antibiotics (streptomycin or gentamicin) display concentration-dependent bactericidal activity against "most gram-negative aerobic and facultative anaerobic bacilli" but not against gram-negative anaerobes and most gram-positive bacteria [3-6]. They act to cause codon misreading by binding to the 30S ribosomal subunit and blocking the translocation of peptidyl-tRNA from the acceptor site to the donor site [7,8].

Penicillin antibiotics may be effective against many bacterial infections caused by staphylococci and streptococci by disruption of peptidoglycan layer in cell wall [9]. Several studies have reported adverse effects of the addition of antibiotics to media upon the rates of embryonic development *in vitro* in human, hamster and mouse, but the underlying causes of this effect have yet to be studied in detail [10-14].

This study analysed the influence of two aminoglycosides and penicillin in the mouse zygote development and then subjected the resulting blastocysts to global gene expression analysis by single embryo RNA-seq. The results show that at the dose commonly used in clinical grade media each class of antibiotic caused pervasive changes in the pattern of gene expression. The predominant effect was for a downregulation of gene expression, and this occurred across a range of regulatory pathways, including those governing the integrity of the genome.

## MATERIALS AND METHODS

### Animal experiments

Animal experiments were conducted with the approval by the ethics committee of Wenzhou medical university. Hybrid (C57BL/6 X CBA/He) mice in were housed and bred in Wenzhou Medical University. Ovulation was induced in six-week-old female mice by intraperitoneal injection of 5 IU Pregnant Mare Serum Gonadotropin (PMSG) (Ningbo Second Hormone Factory, China). After 48 h, mice were intraperitoneal injected with 5 IU human chorionic gonadotrophin. Pregnancy was confirmed by the presence of a copulation plug the following morning. Zygotes were recovered 18 h post-hCG from mated females. In brief, pregnant mice were killed by cervical dislocation. The

oviducts were dissected through the incisions through the abdominal skin and peritoneum. They were transferred into 37°C pre-warmed HEPES-buffered human tubal fluid medium (HEPES-HTF) in 35 mm dishes (Nunclon, Denmark, Cat. 153066), rinsed briefly to remove attached blood and tissues.

Cumulus masses were recovered by tearing the tissue adjacent to the cumulus mass allowing the cells to be expelled into HEPES-HTF. They were then transferred and to 300 IU hyaluronidase (Sigma Chemical Company, St Louis, MO, USA) to remove cumulus cells.

After extensively washed in HEPES-HTF at least 3 times, zygotes were cultured at density of 10 embryos in 10 µl KSOM medium supplemented with 3 mg bovine serum albumin/mL in 60-well plates (LUX 5260, Nunc, Naperville, IL, USA) overlaid with 2 mm of heavy paraffin oil (Sigma) at 37°C in 5% $CO_2$ in air [15]. All components of KSOM medium were embryo culture grade (Sigma).

The working concentrations of three antibiotics (gentamicin sulfate salt (Sigma G-1264), penicillin-G sodium salt (Sigma-P3032, ≥ 1477U/mg) and streptomycin sulfate (Sigma S-9137, ≥ 720 IU/mg)) were prepared by dissolving directly in KSOM medium on the day of use. The treatments were then prepared by a serial dilution in KSOM to produce: (i) control medium without antibiotics; (ii) 0.01, 0.1, 1 mg Gentamicin/mL; (iii) 72 ,720, 7200 IU Penicillin/mL; (iv) 0.05, 0.5, 5 mg Streptomycin/mL. Zygotes were cultured for 96 h to assess their effects on developmental outcomes and gene expression.

## Blastocyst outgrowth

Blastocyst outgrowth was performed as previously described [16]. Normally morphological blastocysts from control and antibiotics treatments were cultured individually in each well of a 24-well plate with 1 mL Dulbecco's Modified Eagles Medium (DMEM, Sigma) supplemented with 10% (v/v) heat inactivated (40 min at 56°C) Fetal Bovine Serum (FBS, Sigma). After 96 h, the area of the epiblast or whole embryo was outlined using the Area of Interest (AOI) for analysis.

## Single blastocyst RNA-seq

Six individual morphologically normal blastocysts from 3 replicates of each treatment with the lowest dose of antibiotics was selected and subjected to RNA-seq. RNA Seq cDNA preparation was as described, with modification [17]. Briefly, each blastocyst was washed in cold PBS and then lysed in the buffer containing 10 X PCR buffer II, 25 mM $MgCl_2$, 10% NP40, 0.1M DTT, 0.5 µM UP1, 2.5 mM dNTPs, SUPERase• In™ RNase Inhibitor and Ambion™ RNase Inhibitor at 70oC for 90s to obtain whole RNA. RNA was reverse transcribed with SuperScript III RT and T4 gene 32 protein to synthesize the first strand cDNAs.

After free primers were removal by Exonuclease I, the 3' end of the first stranded cDNA was added with poly (dA) tail by the reaction with RNase H, 100 mM dAMP, and terminal deoxynucleotidyl transferase. Second strand cDNAs were synthesized by dNTPs, AUP2 primer, TakaRa EX Taq HS and they were 5'-UP2-(T)n-cDNA-(A)n-UP1-3'.

The first amplification of cDNAs was performed with TakaRa EX Taq HS and UP1 primer by 20 cycles. The cDNAs were purified with QIAQuick PCR purification kit and then amplified by second round of 9 cycles PCR with Amine-blocked UP1, amine- blocked UP2 and TakaRa EX Taq HS. After purification of the cDNAs with QIAQuick PCR purification kit, the concentration of cDNAs was measured with Qubit. Approximately 1 ng of this cDNA was processed with TruePrep DNA library Prep Kit V2 for Illumina (Vazyme, Nanjing, China), according to the manufacturer's instructions.

After the fragmentation step of the full-length transcripts, a 10-cycle PCR amplification was performed using a unique combination of index 1 (i7) and index 2 (i5) adapters per library. The average length distribution of the

fragmented cDNA libraries was assessed by the high sensitivity DNA kit (Agilent, USA) and the libraries quantified by Qubit (Thermo Scientific, USA), prior to equimolar pooling.

Libraries were sequenced using Illumina HiSeq X Ten (Illumina, San Diego, CA, USA). All reagents were bought from Thermo Fisher Scientific unless mentioned specifically. The cDNA library reads generated are deposited with the BioProject accession number PRJNA820513. The raw sequencing reads (FASTQ files) were trimmed with Trimmomatic (V0.38) to remove the adapters, discard low-quality reads and obtain the clean reads [18].

The reads were aligned to the reference genome (Mus_musculus. GRCm38) to obtain bam files using HISAT2 (V2.1.0) [19]. FPKM (fragments per kilobase of exon model per million mapped reads) and TPM (transcripts per million) were used for With-sample Normalization. Using StringTie (V1.3.4d) the alignment files were reconstructed and the transcripts were assembled. After initial assembly, the assembled transcripts are merged together, which creates a uniform set of transcripts for all samples [20].

The expression levels of each gene and transcript were quantitated and the differences in expression for all genes among the different experimental conditions were calculated [21]. Transcript_ratio and Gene_ratio was calculated. QualiMap (V2.2.1) was used for the statistical analysis of 5'bias, 3'bias and reads_exonic_ratio, and RSeQC (V3.0.0) for rRNA_ratio.

Differential gene expression between no antibiotic control and each antibiotic treatment was analyzed using the R statistical package DESeq2 (V1.22.2) [22]. Bioinformatic analysis of the Gene Ontology (GO) analysis of differentially expressed genes was performed using the Enrichr tool [23]. On ontologies catalog, the analyzed GO terms included Biological Process 2018 and KEGG 2019 Mouse Pathways (Kyoto Encyclopedia of Genes and Genomes).

## Quantitative Reverse transcription-PCR (qRT-PCR)

QRT-PCR was performed as described previously [24]. Individual blastocysts derived from embryo culture was washed in cold PBS 3 times to remove the media and transferred in minimal volume to 2 µL of RNA buffer containing 0.1 IU RNase inhibitor (Thermo Fisher Scientific). RNA was extracted by three repeats of freezing in liquid nitrogen and thawing with vortex. The RNA was purified with RQ1 RNase-Free DNase Kit (Thermo Fisher).

CDNA synthesized was performed with 1st strand cDNA kit (Thermo Fisher) according to manufactory instruction. Negative controls were either reactions without reverse transcriptase or without the RNA sample replaced by DEPC treated MilliQ water (to test for any RNA or DNA contamination).

An internal positive control was to test for expression of Actb. Amplification of cDNA was performed with sequence specific primers as follows and SYBR Green PCR master mix (Thermo Fisher) on Step One plus Real-Time PCR System (Thermo Fisher). Ct values were calculated by the system software.

The Delta Ct was a measure of relative changes in the transcripts of the tested gene content of the embryo to housekeeper gene Actb. A plot of 2-(normalized DeltaCt) was shown. The PCR products were analysed on 3% agarose gel electrophoresis. Samples of each transcript were sequenced to confirm identity (Table 1).

**Table 1.** The primer sequences for RT-PCR analysis of targeted genes and reference gene.

| Gene name | Gene bank number | Forward | Reverse | Predicted size |
|---|---|---|---|---|
| Actb | NM_007393.5 | GTACCCAGGCATTGCTGACA | AACGCAGCTCAGTAACAGTC | 237 |
| Brca2 | NM_001081001.2 | CCAAAAGATAGGCCTGAGACTTC | TGAGGAAAGCTCCTCAAACCA | 167 |
| Brip1 | NM_178309 | ATAACGCCCGTGCTGTCATA | TTGTGCCGAATACATCTGCC | 187 |
| Rad54l | NM_009015.3 | TGTCTCCTTTTCGGAAGCCC | AGCCCAGGGGACCTTGATAA | 141 |
| Rad51c | NM_001291440.2 | CGCCCAACAAATGATCAGCC | CCCCCAGCTTTCCCCTAATG | 124 |

### Immunofluorescence

Immunofluorescence was performed as previously described [25,26]. After fixation, permeabilization and blocking, embryos were incubated overnight at 4°C with primary antibodies: 2 µg/mL rabbit anti- NANOG polyclonal IgG, 2 µg/mL rabbit anti-BRCA2 polyclonal IgG, 2 µg/mL mouse anti-RAD51C polyclonal IgG and 2 µg/mL isotype negative control immunoglobulin. All primary antibodies were purchased from Abcam (Cambridge, MA, USA).

Primary antibodies were detected by Texas Red- conjugated goat-anti mouse (Sigma) or FITC-conjugated goat anti-rabbit (Sigma) secondary antibodies for 1 h at room temperature. Nuclear localization was determined by staining with 10 µg/mL Hoechst33342 (Sigma) or 0.1 µg/mL Propidium Iodide (PI) (Sigma).

Whole section imaging was captured by NiKon microscope with x 20 len (Nikon, Japan). All quantitative analysis of immunofluorescence experiments was performed with Image-Pro Plus (version 6.3, Media Cybernetics USA). The number of the Inner Cell Mass (ICM) was determined by counting the cells that expressed NANOG, and total cell number by counting of Hoechst33342 stained nuclei in the blastocyst. Optical sectioning was performed with a LEICA SP8 confocal microscope (Leica, Germany) equipped with an APO 63X oil objective.

## RESULTS AND DISCUSSION

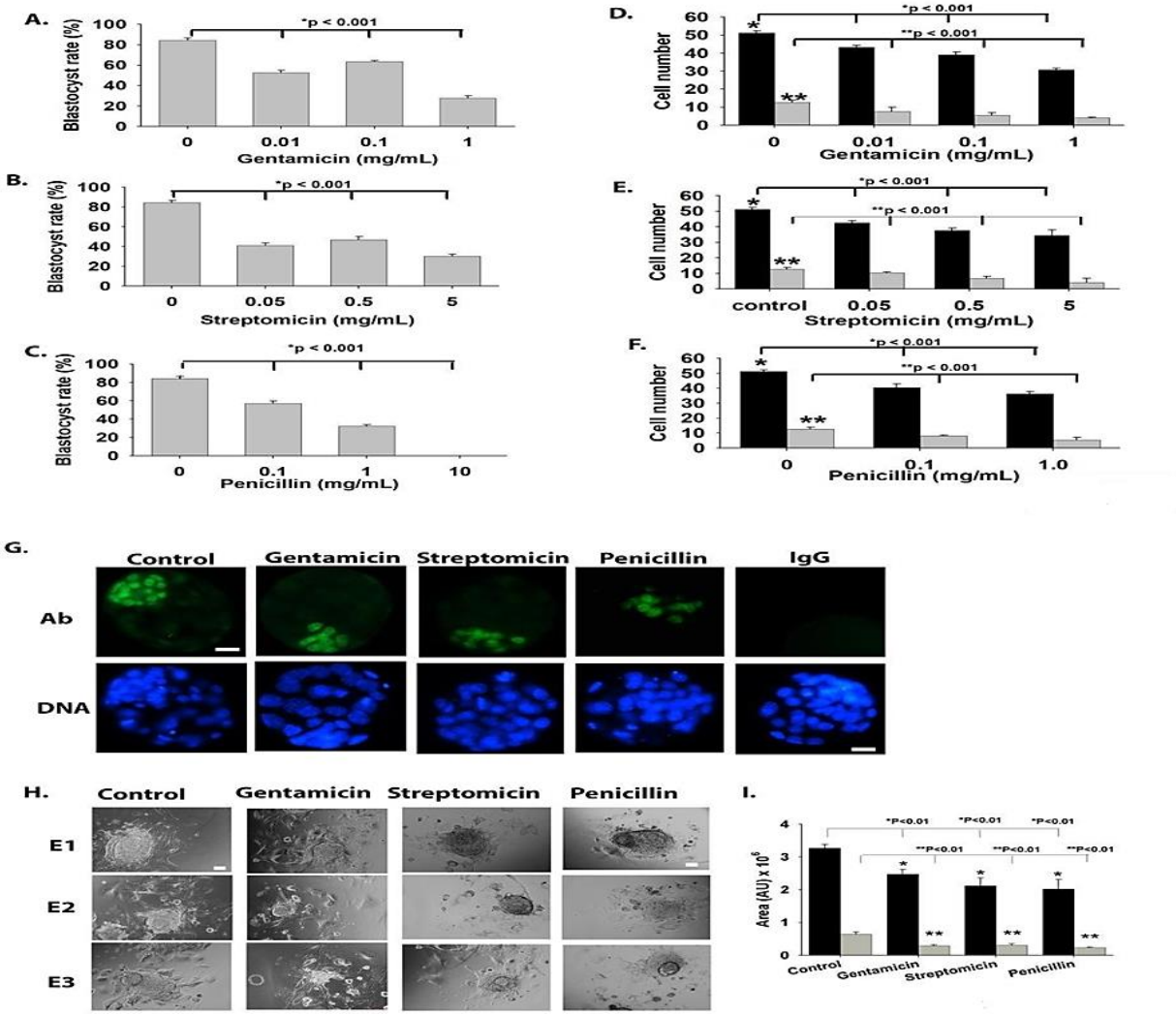
### Antibiotics caused the adverse developmental viability *in vitro*

The zygotes were cultured in the KSOM medium supplemented with a range of 3 doses of each antibiotic. Each treatment caused a dose-dependent reduction in the proportion of zygotes developing to morphological blastocys, and also the total number of cells and the number of NANOG positive pluripotent cells within the Inner Cell Mass (ICM) in the resulting embryos.

The cells expressing NANOG were localized in ICM of morphological blastocysts after antibiotics treatments. These blastocysts had a lower capability of further outgrow 96 h *in-vitro*. The epiblasts were unexpanded and fewer trophoblast cells attached, showing the smaller area of outgrowth and epiblast compared to the control. Negative control was the non-immune IgG instead of antibody (Figures 1A-1l).

**Figure 1.** The effect of antibiotics on mouse zygote development *in vitro*. (A, D) The blastocyst formation rate and the number of total cell and ICM in the blastocysts derived the KSOM medium supplemented with addition of gentamycin; (B,E) streptomycin; (C,F) Penicillin; (G) The normally morphological blastocysts were tested for NANOG; (H) The normally morphological blastocysts were further cultured *in vitro* and 3 embryos (E1-3); (I) Which representative of at least of 20 embryos for each treatment. **Note:** (▬) Total cell; (▬) ICM.

**Significance:** *p<0.001, dose-dependent effect on blastocyst formation rates between the control (without antibiotics) and a range of antibiotic doses. *p<0.001, total number of cells in the blastocyst compared to each dose of the antibiotic. **p<0.001, the number of ICM cells compared to each dose of the antibiotic. *p<0.01 and **p<0.01 each antibiotic compared to the control.



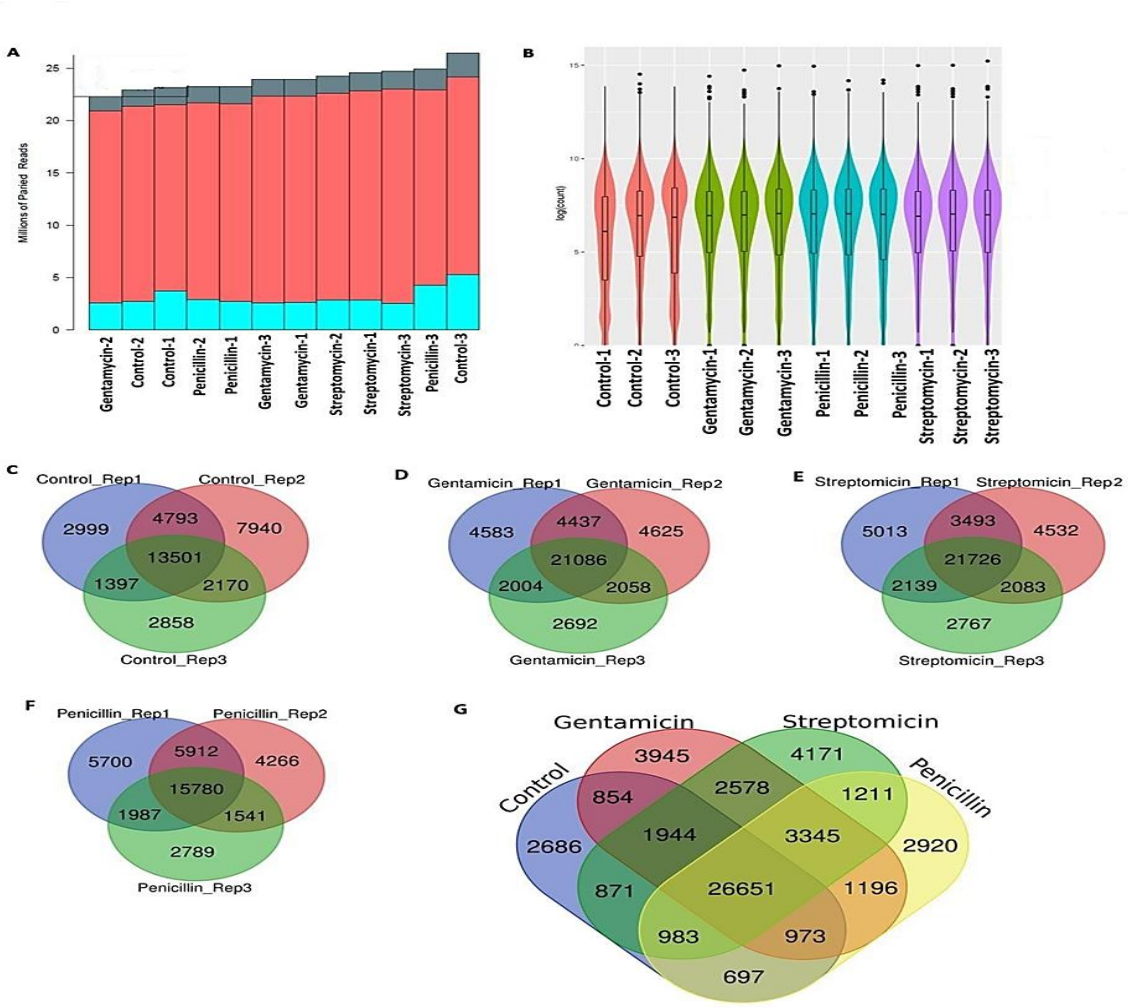

The results were representative of three independent replicates, and each replicate had at least 20 embryos for each dose of antibiotic.

### RNA-seq analysis of antibiotics-related differential expression of transcriptome in the blastocysts

Representative morphologically normal blastocysts from the control and the lowest dose of each antibiotic treatment were selected and subjected to individual embryo RNA-Seq analysis. The cDNA library concentrations from each replicate sample were not significantly different between four treatment groups. Average more than 23 million reads were

produced from each treatment, of which more than 85% could be successfully aligned to the reference mousegenome. The commonly expected duplicates rates were shown [27]. A high ratio of exonic_reads was obtained and almost no rRNA remained in treatment samples, indicating a satisfactory quality of the poly(A) RNA-seq libraries [28,29]. By comparison, the data for negative control (No-RT) produced a low output of cDNA and reads. The data for a range of -5<log 2-fold-change<5 that differed between negative control and no-antibiotic control were considered as noise distribution and ignored for further analysis and thus, the data for $5 \leq \log_2$-fold-change $\leq -5$ with adjusted p-value<0.05 were used for the analysis of differential expression between the control and antibiotics treatments (Figures 2A-2G).

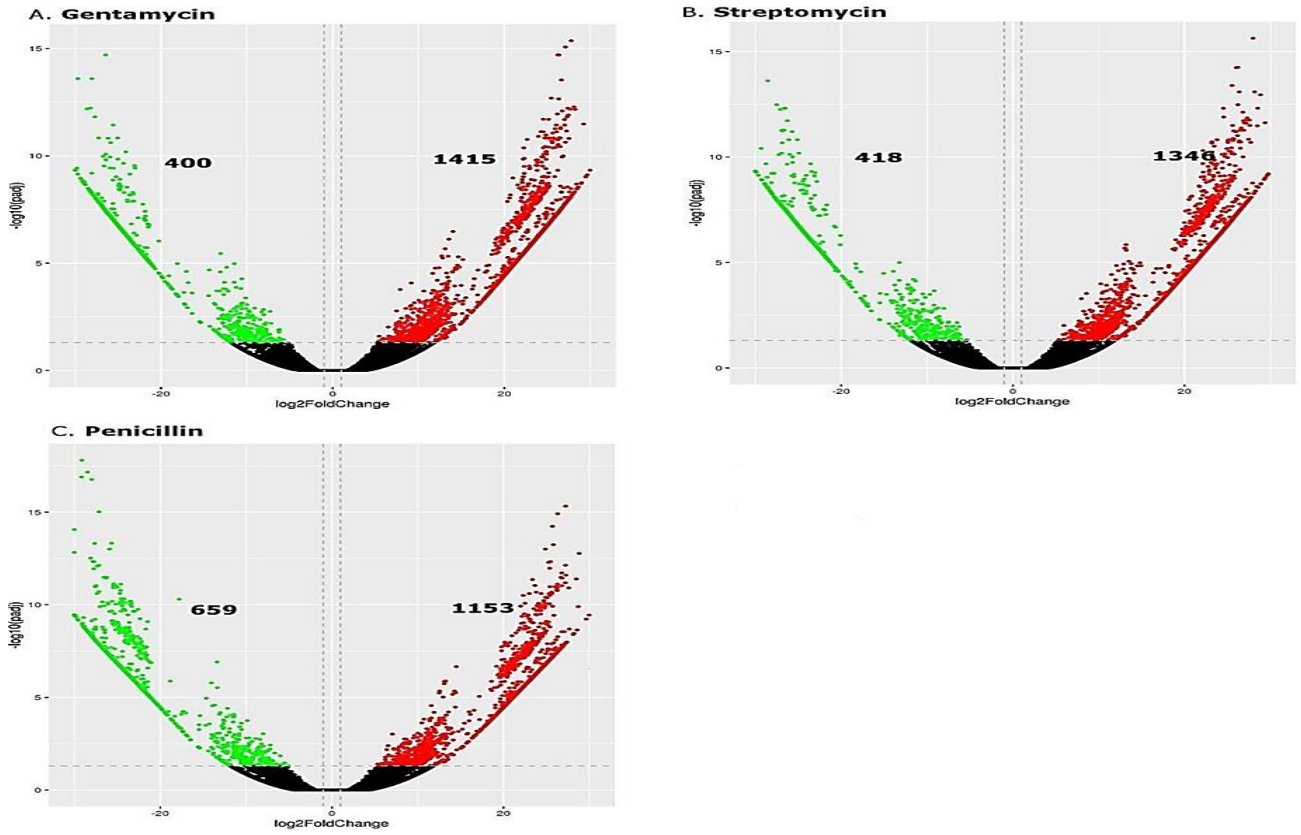
**Figure 2.** RNA-seq analysis of antibiotic-caused DET in single blastocyst. (A) A bar plot showing the number of paired reads to reference transcriptome for each replicate sample; (B) Violin plot showing the Log counts measured to each replicate sample (y-axis: Log10-scaled) across replicates from four treatment groups (x-axis). Violins were grouped and colored by distinct treatment groups of library preparation. Venn diagram illustrating the relationship and overlap of transcripts identified by RNA-seq in the blastocysts derived from the replicates of same treatment; (C) And the antibiotics and control groups; (D) Venn diagram was colored by distinct antibiotics and control treatments. **Note**: (■) Uniquely_Map;( ■) Muliti Map;(■) Unmapped.




Venn diagram analysis demonstrated the more than 47% similarity of transcripts between the replicates of same treatment and the 26651 transcripts were commonly detected between 4 treatment groups while markedly few unique transcripts were found in each treatment group. Comparison of global gene expression in the blastocysts between

controls and each antibiotic treatment showed the differential expression of~1r800 genes accounted for transcriptome change, of which the greater part of genes was down regulated (Figures 3A-3C).

**Figure 3**. The effect of three antibiotics on transcription compared to no treatment control in the blastocyst. (A) Gentamycin; (B) Streptomycin; (C) Penicillin.




The Volcano plot showed the differentially expressed genes according to the p-adj and log 2Fold change values. Distinct colors presented the down- and up-regulated, and unregulated transcripts, and the number of transcripts was shown.

Gentamicin and streptomycin gave 1415 and 1348 downregulated genes, respectively, more than penicillin 1153 genes. Inversely, they caused 400 and 418 upregulated genes, respectively, fewer than penicillin 659 genes. Antibiotics modulated a range of biological processes and KEGG pathways associated with maintenance of genomic integrity. Gene's downregulated as a result of gentamicin treatment was associated with the enrichment of 72 GO Biological Process (BP) and the top 10 included several DNA biological processes, amyloid precursor protein metabolism, protein modification and cell cycle.

Top three BP terms were involved in DNA biological roles, including: DNA metabolic process, DNA recombination and cellular response to DNA damage stimulus. Gentamicin treatment impacted P-value ranked 21 KEGG pathways with the top 10 listed including the cell cycle, mannose type O-glycan biosynthesis, RNA transport, homologous recombination pathways. Genes down regulated in response to streptomycin involved 66 BP and top 10 mainly involved cell cycle and DNA biological processes, including mitotic cell cycle phase transition, DNA metabolic process and cell cycle G2/M phase transition. There were 27 KEGG signal pathways were affected, including cell cycle, tight junction, Homologous recombination, RNA transport; Ubiquitin mediated proteolysis, Mannose type O-glycan biosynthesis, Glycerolipid metabolism and Fanconi anemia pathway. Penicillin downregulated genes affected 51 enriched terms of BP. The top 10 of these included RNA processing, phosphatidic acid biological processing, DNA recombination, cholesterol metabolic

process, cell cycle and vesicle transport along microtubule. 15 KEGG signal pathways were affected, and three of the top 10 KEGG pathways included Glycerophospholipid metabolism, Choline metabolism in cancer, Homologous recombination, glycerolipid metabolism and Mannose type O-glycan biosynthesis. It was noted that there were some similarities on the impacts of all three antibiotic treatments, included two BP enriched terms: DNA metabolic process (GO:0006259) and mitotic cell cycle phase transition (GO:0044772) and two KEGG pathways: "Homologous recombination and, Mannose type O-glycan biosynthesis.

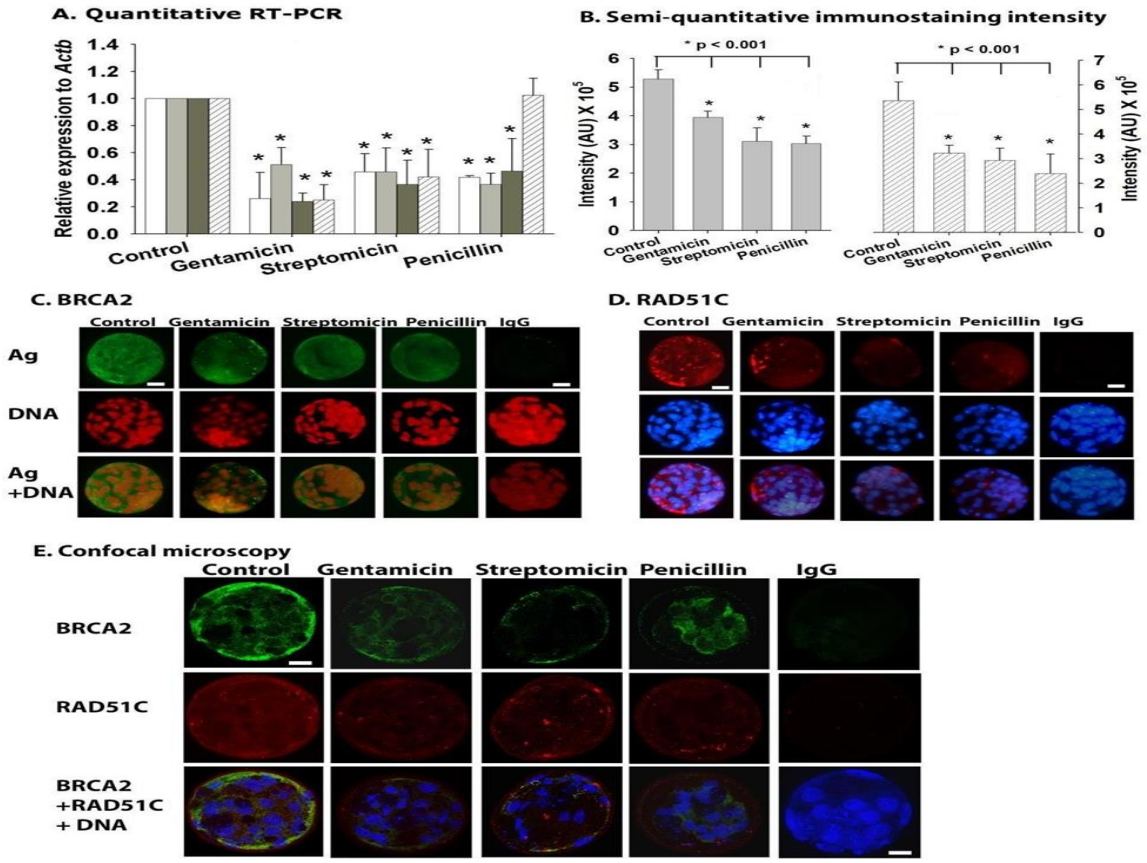
These two KEGG pathways involved two groups of genes: Rad52, Blm, Rad51c, Rad54l, Brcc3, Brca2; B3galnt2, Pomt1, Pomgnt2, Pomgnt1, Large2, respectively. The key differences between the three antibiotics were that Atm was also involved in gentamicin and streptomycin-down-regulated "Homologous recombination", and Uimc1 in penicillin. It is noted that antibiotics caused down-regulation of transcription of a range Trp53-dependent BP enriched terms. All three affected "regulation of signal transduction by p53 class mediator (GO:1901796)", which involved in a range of genes including Plk3, Blm, Meaf6, Taf9, Noc2L, Hipk1, Aurkb, Tpx2, Ubb, Chek1, Taf6, Kmt5a, Rbbp7, Rad9b, Taf4, Rad9a. The key differences were that gentamycin and streptomycin also downregulated Ehmt2, Brip1, Cdk2 and Atm, while Penicillin Prkab2. There were fewer genes up-regulated in response to each of the antibiotics. GO term enrichment analysis of these showed 192 BP terms were affected by gentamicin and top 10 was noted to regulate the roles of protein and ion on the membrane and phosphorylation.

33 KEGG signal pathways were found and the most striking was insulin signal pathway that associated with Raf1, Hras, Phka1 and Phka2. Streptomycin upregulated genes modulated 129 enriched terms of BP including regulation of defense to virus associated with Ppm1b, Pcbp2 and Itch genes, while the genes related to their top 10 BP terms were neither clearly associated with streptomycin- resulted 4 KEGG pathways. 134 BP terms were upregulated by penicillinand 8 of top 10 were involved in the several RNA processing terms. They markedly modulated the splicesome KEGG pathway related to upregulated 11 genes (e.g Ddx5). qRT-PCR and immunostaining down-regulated genes in the blastocyst Quantitative real-time RT-PCR was used to confirm and validate the differential expression of a range of genes detected in the RNA Seq analysis.

7 replicates were tested for stable expression of house-keeper gene Actb in the individual blastocysts that were derived from non-antibiotics control and antibiotics treatments, showing no significant difference of Ct values between control, gentamicin, streptomycin and 280 penicillin (Mean ± STD 32.9 ± 0.56, 32.72 ± 0.20, 33.11 ± 0.43 and 32.41 ± 0.40, respectively), and therefore Actb was used for internal control. The reduced expression of Brca2, Rad51c, Rad54l and Brip1 transcripts in the blastocyst that was produced from culture in antibiotics with the lowest dose tested, compared to the control ($p<0.01$), excepts Brip1 was not affected by penicillin. The results were consistent with the finding from RNA-seq analysis. Immunostaining showed that BRCA2 protein was detected and more accumulated in the cytoplasm of the blastocyst derived in no-antibiotic medium.

Overall, lower level of BRCA2 staining was visualized in the blastocysts from the treatments of three antibiotic and its localization was not characteristic of cytoplasmic accumulation but some in the nuclei as well. RAD51C protein was present in the blastocyst with some accumulated staining in the cytoplasm of the trophectoderm cells and relatively even distribution in the inner cell mass cells. This cytoplasmic staining was less present in the blastocysts from the treatments of three antibiotics, and overall level of the protein was also reduced. Similar results were shown in confocal microscopy of BRCA2 and RAD51C dual staining in blastocysts (Figures 4A-4E).

**Figure 4.** Transcripts and proteins of antibiotics-mediated downregulated genes in the blastocyst. (A) qRT-PCR detected the antibiotics-caused reduction in transcription of Brca2, Rad51c, Rad54l and Brip1 in the mouse *in vitro*-developed blastocyst. The data were representative of the Mean ± STD of normalized ΔΔCt to internal housekeeping gene and non-antibiotics treatment (control) for three independent experiments; (B,C,D) The presented semiquantitative staining intensity and whole-section immunostaining images of BRCA2 and RAD51C in the blastocysts produced in control medium and antibiotics-added medium; (E) The presented confocal immunostaining images were the triple staining of BRCA2, RAD51C and Hoechst nuclear DNA staining, which were representative of at least 20 embryos in control and antibiotic treated embryos from three independent experiments. Significance: *P<0.01 for each antibiotic compared to relevant control, *p<0.001, compared to the control.




In the present study, the culture of mouse zygotes to three different classes of antibiotics, even in the lowest concentration usually used in commercial ART media, gave rise to reduced developmental outcomes to the blastocyst stage and further outgrowth. Analysis of the differential gene expression profile from RNA-seq analysis of single blastocyst found this to be associated with both down- or up-regulation of a range of genes. These involved genes implicated in multiple biological processes and signaling pathways. Of these, the expression of a number of genes associated with maintaining genomic integrity was adversely affected by exposure to antibiotics. Most notably, this included the breast cancer- related gene, Brca2, and a range of other related genes required for the maintenance of genomic integrity. BRCA genes including BRCA1 and BRCA2 play different roles in genome stability [30]. Tested three antibiotics did target Brca2, but not Brca1. They caused the downregulation of Brca2, Blm, Rad51c and Rad54l genes involved DNA homologous recombination pathways and also several p53-dependent genes. Also, it is noticed the different target genes of

antibiotics involved in these pathways. Aminoglycoside antibiotics targeted Atm which is a key are related to the genotoxic response via Atm in as early as in 2- cell stage of embryo and Brip1 that is more likely to increased risk of ovarian cancer. Penicillin downregulated the Uimc1 and Prkab2. Uimc1 (Ubiquitin Interaction Motif Containing 1) interacts with BRCA1 and plays a central role in the BRCA1-A complex to recognize and repair DNA lesions [31].

Prkab2 (Protein Kinase AMP-activated non-catalytic subunit gamma 2) is a downstream gene of Brca2 and helps sense and respond to energy demands within cells in many different tissues [32].

Deletion of Brca2 is embryonic lethality before E 8.5 and the total number of Mouse Embryo Fibroblasts (MEFs) from D 14-16 BrcaTr/Tr mouse is markedly reduced and [33,34]. These MEFs have progressive proliferative impairment and replicative failure in culture. Present study found three tested antibiotics caused the retarded proliferation of blastocysts during further outgrowth *in vitro*. These impacts for the proliferation of extraembryonic tissues may be evident to the implantation failure in assisted reproductive technologies. BRCA2 physically and functionally interacts with $P^{53}$ and RAD51 and is implicated in the regulation of cell cycle and DNA repair pathways [35]. Antibiotics altered the immuno-detectable levels of BRCA2 and RAD51C in the embryo, whereas during IVF, a range of stressors associated with embryo culture increased the levels and nuclear localization of $P^{53}$, resulting in reduced rates of development and poor post implantation rates of survival [36]. Conversely, embryo trophic ligands acting *via* a phosphatidylinositol-3-kinase/AKT/MDM2 signaling pathway repressed the level of $P^{53}$ within embryos [37]. Several epidemiological studies have suggested a positive association between clinical use of antibiotic and the risk of breast cancer, Rossini tumor and antibiotics metronidazole/cip but not gentamycin, whereas gentamicin may arrest cancer cell growth [38-41]. The present study for the first time demonstrates association of antibiotics with the breast cancer related genes in the preimplantation embryo development *in vitro*. $BRCA^{1/2}$ are tumor suppressor genes. $BRCA^{1/2}$ mutations are embryo-lethal and are associated with the lower ovarian reserve [42,43].

The BRCA mutation has been detected in IVF cycles by Preimplantation Genetic Diagnosis (PGD) since 2008. PGD is a universal test for BRCA mutation carriers who require ART and fertility preservation [44-46]. More recent evidences of BRCA mutation in oocyte aging and sperm aging [47,48]. However, the incidents of intragenic $Brca^{1/2}$ mutations between natural reproduction and ART are still lacking as ethical complexities of PGD are concerned. If the adverse effect of antibiotics on these tumor suppressor genes also occurs in human embryos created by ART, it is conceivable that these procedures may influence the inheritance of such deleterious mutations. The outcomes of likely increased rates of Brca2-related disorders in ART generations requires for the detailed investigation. This study showed most of the changes observed was due to downregulation instead of upregulation. It did not address the potential mechanisms, but may indicate that a transcriptional process common to many gene targets is adversely affected by these treatments. Global profiles of gene expression in bacteria Acinetobacter oleivorans showed three antibiotics (ampicillin, tetracycline, norfloxacin) caused more percentage of downregulated genes and one antibiotics (kanamycin) more upregulated [49]. This study shows that more investigation into the underlying biology of the effects of antibiotics on embryos *in vitro* is a priority.

Common concentrations in commercial ART embryo media are 100 IU Penicillin, 50-100 µg streptomicin/mL and 10-50 gentamicin µg/mL. Penicillin and streptomycin reduced human embryo cleavage rate and prevented gene expression for mouse zygotic gene activation. Streptomycin resulted in a significantly lower fertilization rate and the blastocyst formation rate. It is noted these concentrations in ART media may produce the embryos with normal appearance, but possess intrinsic physiological and genetic abnormalities.

## CONCLUSION

In this study, these also caused instability of Brca2-regulated genomic integrity which resulted in the failure of post-implantation embryogenesis. This study provides evidence for pervasive effects of commonly used antibiotics on the pattern of gene expression in the mouse embryo cultured *in vitro*. These changes occurred within many important regulatory pathways, but notably involve many genes performing critical functions and ensuring the integrity of the embryo's genome. Given the centrality of DNA integrity to the future health of progeny resulting from ART procedures a detailed understanding of the potential adverse effect of this media component is an important priority for future research.

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### Declaration of interest

There are no conflicts of interest to declare that could be perceived as prejudicing the impartiality of the research reported.

### Author contribution statement

Conception and design of experiments: X.H, C.O, and X. Jin. Experiments performed by: Q.H, X.J, Y.L, L.C, J.S, W.N, W.L and X.Jin. Data analysis performed by: Q.H, X.H and X.Jin. Reagents, materials, analysis tool contribution: Y.L, J.L, X.H and X.J. Manuscript written by: C.O and X.Jin. All authors agreed to the publication.

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