1   **Iodoacetic acid disrupts mouse oocyte maturation by inducing oxidative stress**
2   **and spindle abnormalities**

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23   **Abstract**

24   Disinfection by-products (DBPs) are compounds produced during the water
 25   disinfection process. Iodoacetic acid (IAA) is one of the unregulated DBPs in
 26   drinking water, with potent cytotoxicity and genotoxicity in animals. However,
 27   whether IAA has toxic effects on oocyte maturation remains unclear. Here, we show
 28   that IAA exposure resulted in metaphase I (MI) arrest and polar-body-extrusion
 29   failure in mouse oocytes, indicating that IAA had adverse effects on mouse oocyte
 30   maturation *in vitro*. Particularly, IAA treatment caused abnormal spindle assembly
 31   and chromosome misalignment. Previous studies reported that IAA is a known
 32   inducer of oxidative stress in non-germline cells. Correspondingly, we found that IAA
 33   exposure increased the reactive oxygen species (ROS) levels in oocytes in a dose-
 34   dependent manner, indicating IAA exposure could induce oxidative stress in oocytes.
 35   Simultaneously, DNA damage was also elevated in the nuclei of these IAA-exposed
 36   mouse oocytes, evidenced by increased γ -H2AX focus number. In addition, the un-
 37   arrested oocytes entered metaphase II (MII) with severe defects in spindle
 38   morphologies and chromosome alignment after 14-hour IAA treatment. An
 39   antioxidant, N-acetyl-L-cysteine (NAC), reduced the elevated ROS level and restored
 40   the meiotic maturation in the IAA - exposed oocytes, which indicates that IAA-
 41   induced maturation failure in oocytes was mainly mediated by oxidative stress.
 42   Collectively, our results indicate that IAA exposure interfered with mouse oocyte
 43   maturation by elevating ROS levels, disrupting spindle assembly, inducing DNA
 44   damage, and causing MI arrest.

45 **Key words:** Iodoacetic acid, mouse oocyte, oxidative stress, spindle assembly, DNA
46 damage

47 **1. Introduction**

48 Water disinfection byproducts (DBPs) are types of by-products produced during water
49 disinfection processes that are designed to prevent the outbreak of waterborne
50 diseases. DBPs can be generated from the reaction of disinfectants with organic or
51 inorganic materials including bromide, chloride, and iodide in raw water (Fabbricino
52 and Korshin, 2009; Farré et al., 2013). Currently, more than 700 DBPs have been
53 detected in drinking water. Humans and non-human animals can be exposed to DBPs
54 through various routes including inhalation, ingestion, and dermal absorption
55 (Gonsioroski et al., 2020b; Teuschler et al., 2004). Previous studies reported that
56 DBPs can exert adverse effects, such as cytotoxicity, genotoxicity, mutagenicity, and
57 carcinogenicity (Richardson et al., 2007). Epidemiological studies indicate that DBP
58 exposure is associated with the increased risk of cancers, including bladder cancer
59 (Villanueva et al., 2004), colorectal cancer (Rahman et al., 2010), and rectal cancer
60 (Jones et al., 2019). In addition, DBP exposure is thought to cause reproductive
61 dysfunction (Gonsioroski et al., 2020a; Jeong et al., 2016), developmental toxicity
62 (Andrews et al., 2004; Ding et al., 2020), and adverse pregnancy outcomes (Costet et
63 al., 2012; Levallois et al., 2012; Toledano et al., 2005; Wright et al., 2017). The
64 United States Environmental Protection Agency (U.S. EPA) has regulated the levels
65 of 11 DBPs in drinking water due to the rising of health concerns (USEPA 2006).
66 However, a large number of DBPs in drinking water are unregulated, likely due to a

67 lack of studies on their potential toxicity.

68 Iodoacetic acid (IAA), an unregulated DBP in drinking water, forms during
69 chlorinated disinfection of raw water. IAA is now attracting more attention due to its
70 ubiquity and high toxicity. Richardson et al (2008) showed that the maximum level of
71 IAA is up to 1.7 µg /L in water samples from 23 cities in the United States and
72 Canada. Another study detected a high maximal level of IAA (2.18 µg/L) in drinking
73 water in China (Wei et al., 2013).

74 In addition to being a ubiquitous pollutant, IAA is thought to be toxic in many
75 systems. Wei et al (2013) showed that IAA exposure reduced cell viability in NIH3T3
76 cells at a concentration as low as 2.5 µM, and increased DNA damage in a
77 concentration-dependent manner. Other studies also reported that IAA induces
78 cytotoxicity and genotoxicity in CHO cells (Plewa et al., 2010), primary human
79 lymphocytes (Escobar-Hoyos et al., 2013), and HepG2 cells (Wang et al., 2014;
80 Zhang et al., 2012).

81 The mechanism by which IAA causes toxicity is thought to involve oxidative
82 stress. For example, two antioxidants, catalase and butylated hydroxyanisole (BHA),
83 reduced the percentage of IAA-induced genomic DNA damage in CHO cells by 86.5%
84 and 42%, respectively (Cemeli et al., 2006). Antioxidants also attenuated IAA-
85 induced cytotoxicity and genotoxicity in HepG2 cells (Wang et al., 2014) and primary
86 cerebellar granule neurons CGNs (Zhou et al., 2015).

87 It is also worth noting that IAA may pose risks to female reproduction. Recent
88 studies showed that IAA exposure inhibited follicle growth through altering the

89 expression of the genes that regulate the cell cycle, apoptosis, estrogen receptors
 90 (ERs), and ovarian steroidogenesis (Gonsioroski et al., 2020a; Jeong et al., 2016).
 91 However, whether IAA exposure affects oocyte meiotic maturation has not been
 92 explored. Thus, in the present study, we utilized an *in-vitro* mouse oocyte culture
 93 system to study the effects of IAA exposure on oocyte maturation *in vitro*. Our results
 94 showed that IAA exposure arrested mouse oocytes at the metaphase I stage, leading to
 95 polar body extrusion (PBE) failure. IAA also increased DNA damage and disrupted
 96 spindle assembly and chromosome alignment in treated oocytes. Since IAA can
 97 induce oxidative stress in non-germline cells, we tested whether IAA-induced
 98 oxidative stress disrupts oocyte maturation. We found that IAA caused oxidative
 99 stress in mouse oocytes, which was evidenced by an elevated ROS level in response
 100 to IAA. To confirm whether oxidative stress is the major cause of the oocyte arrest,
 101 we added an antioxidant, N-acetyl-L-cysteine (NAC) in the culture media. We found
 102 that NAC rescued IAA-induced maturation failure. Collectively, the data from this
 103 study provide new information on the toxicological mechanisms underlying the
 104 effects of IAA on oocyte maturation and raise concerns that IAA exposure poses risks
 105 to reproductive health.

106 **2. Materials and methods**

107 **2.1 Chemicals**

108 Iodoacetic acid ($\geq 98\%$), 3-Isobutyl-1-methylxanthine (IBMX, $\geq 99\%$), N-acetyl-L-
 109 cysteine (NAC, $\geq 99\%$) and 2',7'-dichlorofluorescein diacetate (DCFH-DA, $\geq 97\%$)

110 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pregnant mare serum
111 gonadotropin (PMSG) was obtained from ProSpec TechnoGene.

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113 **2.2 Animals**

114 CD-1 mice (Charles River Laboratories, Wilmington, MA) were used in this study
115 and housed in the Animal Care Facility at the University of Illinois Urbana-
116 Champaign (UIUC). Mice were housed under 12 h dark/12 h light cycles at $22 \pm 1^\circ\text{C}$
117 and were provided with food and water ad libitum. Animal handling and procedures
118 were approved by the UIUC Institutional Animal Care and Use Committee.

119 **2.3 Oocyte collection and culture**

120 Three-week-old female mice were injected with 5 IU PMSG to stimulate follicular
121 development. The treated mice were euthanized 48 h after PMSG treatment.
122 Cumulus-oocyte complexes (COCs) were isolated by rupturing antral ovarian
123 follicles with sterile needles. Cumulus cells around the fully-grown oocytes were
124 mechanically removed as previously described (Qiao et al., 2018). The isolated
125 oocytes were collected in M2 medium (Sigma-Aldrich, St. Louis, MO, USA)
126 containing 100 μM IBMX. After three washes in fresh M16 medium, oocytes were
127 cultured in a drop of pre-warmed M16 medium (Sigma-Aldrich, St. Louis, MO, USA)
128 under mineral oil at 37°C in a 5% CO_2 incubator. After culture in maturation medium
129 for 2 h, 8 h, 10.5 h, and 14 h, oocytes progressed to germinal vesicle breakdown
130 (GVBD), metaphase I (MI), anaphase I (AI)/telophase I (TI), and metaphase II (MII),

131 respectively. Then, oocytes were collected at each stage for data analysis.

132 **2.4 IAA and NAC treatment**

133 The concentration of IAA was chosen based on our previous study (Gonsioroski et al.,
134 2020a). IAA (10 μ M and 15 μ M) was found to significantly decrease follicle growth
135 *in vitro*. Thus, we speculated that oocyte maturation may also be affected by IAA
136 treatment because follicles are composed of oocytes and granulosa cells. IAA was
137 dissolved in dimethyl sulfoxide (DMSO) and diluted in maturation medium (M16)
138 with final concentrations of 2 μ M, 5 μ M, and 10 μ M. The final concentration of the
139 solvent DMSO was no more than 0.1% in all culture media. Oocytes in the control
140 group were cultured in M16 with the same amount of DMSO, but no IAA. An
141 antioxidant, NAC supplementation, was used to determine whether it rescues the
142 damage induced by IAA. The concentration of NAC was based on a previous study
143 by Lai et al. (2015). NAC was dissolved in M16 medium and diluted into the
144 maturation medium with or without IAA at a final concentration of 200 μ M.

145 **2.5 Immunofluorescent staining**

146 Oocytes were fixed in PBS (pH 7.4) containing 4% paraformaldehyde for 30 min at
147 room temperature. After the oocytes were washed in washing buffer (0.1% tween-20
148 and 0.01% triton X-100 in PBS) twice, oocytes were permeabilized in 0.5% triton X-
149 100 in PBS for 20 min at room temperature. After two washes, the oocytes were
150 blocked in washing buffer with 3% BSA for 1 h at room temperature and then
151 incubated overnight at 4°C with FITC-labelled mouse anti- α -tubulin (1:200, Sigma-

152 Aldrich) or mouse monoclonal anti- γ -H2AX antibody, clone JBW301 (1:200,
153 Millipore-Sigma) and anti-phospho-p44/42 MAPK (Erk1/2) (1:200, Cell Signaling
154 Technologies). As for γ -H2AX or phospho-p44/42 MAPK (Erk1/2) staining, the
155 oocytes were washed in washing buffer for three times (10 min each time), then
156 incubated with the Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) and
157 Alexa Fluor 594-conjugated goat anti-rabbit IgG (H + L) secondary antibodies (1:300,
158 Molecular Probes) for 1 h at 37°C, respectively. Then, oocytes were washed another
159 three times (10 min each time). Oocyte DNA was counterstained with 1 μ g/ml of 4',6-
160 diamidine-2'-phenylindole dihydrochloride (DAPI) for 10 min at room temperature.
161 After DAPI staining, oocytes were mounted on glass slides with 80% glycerol,
162 examined with a Nikon A1R confocal microscope, and processed using NIS-Elements
163 software.

164 **2.6 Measuring the levels of intracellular reactive oxygen species (ROS)**

165 To measure the ROS levels in the control and IAA-treated oocytes, 2',7'-
166 dichlorofluorescein diacetate (DCFH-DA) was used. Oocytes were incubated in M2
167 medium with 4 μ M DCFH-DA for 30 minutes at 37°C in a 5% CO₂ incubator. Then,
168 oocytes were washed twice in PBS and the fluorescent signal was examined
169 immediately by fluorescence microscope (Olympus, IX73). To quantify the oocyte
170 fluorescence intensity among different treatment groups, the images of both control
171 and IAA-treated oocytes were taken using the same imaging setup. The Image J
172 software (National Institutes of Health, USA) was used to measure the fluorescent
173 intensity of the regions of oocytes in these images.

174 **2.7. Statistical analysis**

175 Each treatment was performed at least three replicates. Data are presented as mean $\pm$
 176 SEM. For comparisons between two groups, the analysis was performed using the
 177 GraphPad Prism (San Diego, USA) software followed by Student's unpaired two-
 178 tailed t-test. Comparisons between the three groups were analyzed by one-way
 179 analysis of variance (ANOVA). Specifically, multiple comparisons between normally
 180 distributed experimental groups were performed using ANOVA followed by Tukey
 181 post-hoc comparison. Multiple comparisons between non-normally distributed
 182 experimental groups were performed using the Kruskal-Wallis test. Comparisons were
 183 considered significant at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

184 **3. Results**

185 **3.1. Mouse oocytes failed to mature after IAA exposure**

186 To test the effects of IAA exposure on mouse oocyte maturation, the oocytes were
 187 cultured in media supplemented with increasing concentrations of IAA (2 μ M, 5 μ M,
 188 and 10 μ M). After 2-h or 14-h *in vitro* culture, the GVBD rate and PBE rate were
 189 recorded, respectively. As shown in Figure 1A and B, IAA exposure at all three
 190 concentrations (2 μ M, 5 μ M, and 10 μ M) had no effect on the GVBD rate compared
 191 to control ($P > 0.05$). However, when we examined the effects of IAA exposure on
 192 PBE after 14-h culture, we found that the PBE rates of mouse oocytes were
 193 significantly reduced in the 5 μ M treatment group ($39.83 \pm 5.54\%$) and 10 μ M
 194 treatment group ($22.11 \pm 4.69\%$) compared to the control group ($77.57 \pm 6.87\%$) ($P <$

0.001; Fig. 1 A and C). Although the difference was not significant when compared with control, PBE decreased even when the IAA concentration was at 2 μ M ($70.05 \pm 5.38\%$, $P = 0.14$). Besides, we found that this block was permanent because all of the arrested oocytes in the 14-h-culture 5- μ M-IAA treatment group (Fig. 1A) still failed to extrude their polar bodies at 24 h after an additional 10-h IAA treatment, as shown in Figure 1D. Taken together, these observations revealed that IAA exposure blocked meiotic progression in mouse oocytes, which led to the failure of oocyte maturation. Since 5 μ M and 10 μ M IAA treatments could significantly reduce the PBE rate in mouse oocytes, these two concentrations were used for subsequent analysis to determine how IAA disrupts the oocyte maturation.

3.2. IAA exposure induced metaphase I arrest in mouse oocytes.

Since IAA exposure had no effects on the progression of GVBD, we hypothesized that IAA exposure might result in MI arrest that finally triggered PBE failure. To determine whether the IAA induces MI arrest, we examined meiotic progression with or without IAA (5 μ M and 10 μ M) treatment. To stage the cultured oocytes, we immunostained the oocytes with anti- α -tubulin and counter-stained with DAPI. After 10.5-h culture (2 h for GVBD + 8.5 h), most of the cultured oocytes progressed to anaphase/telophase I (A/TI) in the untreated control group (Fig. 2A). Remarkably, the proportion of MI oocytes in the 5 μ M and 10 μ M IAA-treated groups was significantly higher than that in control at 10.5 h (5 μ M, $P < 0.001$ and 10 μ M, $P < 0.001$, Fig. 2B). On the contrary, the proportion of A/TI oocytes in IAA-treated group was significantly lower than control (5 μ M, $P < 0.001$ and 10 μ M, $P < 0.001$, Fig. 2B).

217 This cell cycle analysis indicated that IAA exposure caused the oocytes to arrest at the
218 MI stage. Further, our data also showed that IAA exposure led to MI arrest in a dose-
219 dependent manner (Fig. 2B). In addition, this MI arrest was also verified by inverted
220 microscopy. After 10.5-h culture, we found that over half of the control oocytes were
221 at telophase I when polar body extrusion was processing, but only a few oocytes with
222 forming polar bodies were found in the IAA-treated groups (Fig. 2C).

223 **3.3. IAA exposure caused abnormalities of spindle assembly and chromosome** 224 **alignment in MI mouse oocytes**

225 To understand how IAA induces PBE failure in oocytes, we investigated spindle
226 assembly and chromosome alignment during MI because these processes are required
227 for PBE. Unexpectedly, spindle assembly was not extremely disrupted by IAA (5 μ M
228 and 10 μ M) exposure as evidenced by a barrel-shaped spindle (Fig. 3A). Next, we
229 measured the spindle length and width of oocytes in control, 5 μ M and 10 μ M IAA-
230 treated groups. IAA exposure significantly increased spindle length in a dose-
231 dependent manner (36.64 ± 4.32 μ m in 5 μ M IAA; 39.98 ± 4.92 μ m in 10 μ M IAA
232 versus 32.03 ± 3.58 μ m in control, $P < 0.001$; 5 μ M IAA versus 10 μ M IAA, $P < 0.01$;
233 Fig. 3B), and it significantly decreased spindle width (17.95 ± 1.82 μ m in 5 μ M IAA,
234 $P < 0.01$; 17.48 ± 2.57 μ m in 10 μ M IAA, $P < 0.001$ versus 19.55 ± 1.91 μ m in
235 control; Fig. 3C). In addition, 5 μ M and 10 μ M IAA did not induce severe dispersed
236 distribution of chromosomes as often found in arrested oocytes (Fig. 3A). However,
237 misalignment of meiotic chromosomes was observed after we measured the MI-plate
238 width. We found that IAA induced a significant increase in MI-plate width compared

239 to the control ($12.07 \pm 2.45 \mu\text{m}$ in $5 \mu\text{M}$ IAA, $P < 0.01$; $13.59 \pm 2.80 \mu\text{m}$ in $10 \mu\text{M}$
 240 IAA, $P < 0.001$ versus $10.71 \pm 1.86 \mu\text{m}$ in control; Fig. 3D). In addition, we found
 241 that a high IAA concentration ($30 \mu\text{M}$) caused severe defects of spindle formation and
 242 chromosome alignment in treated oocytes (Fig. S1). Particularly, spindles in the 30-
 243 μM -IAA-treated oocytes were extremely long and disorganized, which was evidenced
 244 by the mislocalization of p-ERK1/2, microtubule organizing centers (MTOCs)
 245 associated proteins that enrich at spindle poles to facilitate proper spindle formation
 246 (Fan and Sun, 2004).

247 **3.4. IAA exposure increased the level of reactive oxygen species (ROS) in mouse** 248 **oocytes.**

249 IAA was reported to induce an elevation of ROS, leading to oxidative stress in
 250 mammalian cells (Wang et al., 2014; Zhou et al., 2015), and oxidative stress is well-
 251 known to be detrimental to oocyte maturation (Combelles et al., 2009). Thus, we
 252 hypothesized that IAA exposure results in increased ROS levels in mouse oocytes,
 253 which may lead to the aforementioned defects. To test our hypothesis, we used an
 254 oxidation-sensitive fluorescent probe, DCFH-DA, to monitor the generation of
 255 intracellular ROS in mouse oocytes. Our results showed that the DCFH-DA signals in
 256 $5 \mu\text{M}$ and $10 \mu\text{M}$ IAA-treated oocytes were brighter than in control oocytes (Fig. 4A).
 257 Our quantitative analysis verified the observation: the fluorescence intensity of
 258 DCFH-DA in $5 \mu\text{M}$ and $10 \mu\text{M}$ IAA treated oocytes was significantly higher than that
 259 in control oocytes ($P < 0.001$, Fig. 4B). Meanwhile, IAA raised intracellular ROS
 260 levels in oocytes in a dosage-dependent manner, the DCFH-DA intensity in the $10 \mu\text{M}$

261 IAA-treated oocytes was significantly higher than in the 5 μ M group ($P < 0.05$, Fig.
262 4B).

263 **3.5. IAA exposure induced DNA damage in mouse oocytes.**

264 ROS can drive genomic instability and elevated ROS levels usually lead to DNA
265 damage (Salehi et al., 2018; Srinivas et al., 2019). Thus, we hypothesized that IAA
266 exposure results in DNA damage in mouse oocytes due to an increase of cellular ROS
267 levels after IAA exposure. We further checked the DNA double-strand breaks (DSBs)
268 in GV-arrested oocytes using γ -H2AX staining. IBMX was used to arrest the oocytes
269 at the GV stage for 8 h in the three treatment groups (5 μ M IAA, 10 μ M IAA, and the
270 untreated control). Our results show that more γ -H2AX foci were found in 5 μ M and
271 10 μ M IAA-treated oocytes compared to the control (Fig. 5A). Next, comparison
272 between the three groups was made using the Kruskal-Wallis test, and our results
273 showed that the average focus number in 5 μ M and 10 μ M IAA-treated oocytes was
274 significantly higher than in control (2.94 ± 1.96 in 5 μ M, $P < 0.05$; 4.16 ± 2.10 in 10
275 μ M, $P < 0.01$ versus 1.48 ± 1.50 in control) (Fig. 5B). Meanwhile, IAA induced
276 oocyte DNA breaks in a dose-dependent manner and the average foci number in 10
277 μ M IAA-treated oocytes was significantly higher than in the 5 μ M group ($P < 0.05$,
278 Fig. 5B).

279 **3.6. IAA exposure impaired spindle formation and chromosome alignment in** 280 **metaphase II oocytes**

281 After IAA exposure, some oocytes could still undergo PBE. To check the quality of

these surviving oocytes, we examined the spindle morphologies of MII oocytes in the 5 μ M IAA-treated group. Our results showed that most of the MII oocytes in the control group displayed a typical spindle shape and well-aligned chromosomes, whereas many of the 5 μ M IAA-treated MII oocytes had abnormal spindle morphologies and misaligned chromosomes (Fig. 6A). Statistically, the proportion of oocytes with abnormal spindles in the IAA-treated group was significantly higher than that in the control ($39.28 \pm 4.27\%$ in 5 μ M IAA versus $15.68 \pm 9.25\%$ in control, $P < 0.01$; Fig. 6B). In addition, the proportion of oocytes with misaligned chromosomes in the IAA-treated group was significantly higher than that in control ($32.10 \pm 6.36\%$ in 5 μ M IAA versus $13.39 \pm 4.90\%$ in control, $P < 0.01$; Fig. 6C).

3.7. An antioxidant, N-acetyl-L-cysteine (NAC), improved the meiotic maturation rate in IAA-exposed oocytes.

To investigate whether IAA-induced oxidative stress can be alleviated by an antioxidant, NAC (200 μ M), we examined ROS levels in the control, 5 μ M, and 10 μ M IAA-treated oocytes with and without NAC treatment. We found that NAC significantly reduced the elevated ROS level in the 5 μ M and 10 μ M IAA-treated oocytes (5 μ M, $P < 0.01$ and 10 μ M, $P < 0.001$, Fig. 7A and B). Subsequently, the PBE rates were analyzed in all treatment groups to test whether the antioxidant NAC can further rescue the PBE failure induced by IAA exposure. We observed that more oocytes successfully extruded the polar bodies in NAC + IAA co-treated groups compared to the IAA-only groups (Fig. 7C). We also found the PBE rates in the 5 μ M and 10 μ M IAA groups were significantly lower than in the corresponding NAC +

304 IAA groups ($P < 0.01$, Fig. 7D).

305 4. Discussion

306 Recent studies showed that IAA, an unregulated water disinfection by-product,
 307 inhibited antral follicle growth *in vitro* (Gonsioroski et al., 2020a; Jeong et al., 2016),
 308 which suggests that IAA is a reproductive toxicant. This is a concern because female
 309 fertility is declining in the United States (Hamilton et al., 2006) and many
 310 environmental toxicants have been identified as exogenous factors that are associated
 311 with a decline of female fertility (Hunt and Hassold, 2008; Rattan et al., 2017; Sifakis
 312 et al., 2017). Particularly, oocytes are susceptible to chemical-induced perturbations.
 313 Oogenesis along with follicle development produces oocytes that are essential for
 314 female reproduction, but little was known about the effects of IAA on oocyte
 315 maturation. Thus, to our knowledge, this is the first study to explore IAA effects on
 316 oocyte maturation as well as the possible mechanisms underlying IAA toxicity on
 317 oocytes *in vitro*. We have documented the occurrence of PBE failure, abnormal
 318 spindle assembly, chromosome misalignment, ROS level elevation, and DNA damage
 319 in mouse oocytes in response to IAA exposure, and uncovered that the toxic effects
 320 were mainly mediated by IAA induced-oxidative stress.

321 GVBD and PBE are two markers of meiotic progression during oocyte *in-vitro*
 322 maturation (IVM). The first step of IVM is isolating oocytes from antral follicles. The
 323 isolated oocytes are at the germinal vesicle (GV) stage and they will resume meiosis
 324 after *in-vitro* culture. The germinal vesicle/nuclear envelope will subsequently

breakdown. This GVBD process can be morphologically characterized by microscopy (Pan and Li, 2019). When oocytes progress from GVBD to metaphase I stage, the connections between spindle and chromosomes are well established. After the segregation of homologous chromosomes, the oocyte will extrude the first polar body and quickly arrest at the metaphase II stage where the oocyte can be fertilized. Therefore, the extrusion of the first polar body usually marks oocyte maturation (Sánchez and Smitz, 2012). Defects in GVBD will affect the subsequent meiotic progression, which could finally delay or block oocyte maturation (Jiao et al., 2017; Zhang et al., 2020). Another timepoint that can determine oocyte maturation is the metaphase I to anaphase I (MI-AI) transition. Abnormal meiotic events during MI would lead to MI arrest and subsequently inhibit the extrusion of the polar body. Our results showed that IAA did not affect the GVBD rate at any treatment concentrations (Fig. 1B), suggesting that IAA is not toxic to GVBD in mouse oocytes at the selected concentrations. However, IAA (5 μ M and 10 μ M) significantly reduced PBE rates after 14-h culture in a concentration-dependent manner (Fig. 1C), indicating that IAA is toxic to PBE in oocytes at the selected doses. Since GVBD was not disturbed by IAA, we hypothesized that IAA might arrest most of the oocytes at MI, even though a few of oocytes would proceed to the MII stage. Cell cycle analysis supported our hypothesis because more IAA-treated oocytes arrested at the MI stage compared to the untreated control (Fig. 2). These arrested oocytes in the IAA (5 μ M)-treated group could not progress further to undergo PBE at 24 h. Taken together, IAA-exposure induced MI arrest that finally resulted in the failure of oocyte maturation.

347 Proper spindle assembly is essential for chromosome segregation, oocyte
348 maturation, and subsequent embryo development (Howe and FitzHarris, 2013). Our
349 results showed that IAA (5 μ M and 10 μ M) exposure did not cause severe disruption
350 of spindle assembly in oocytes as evidenced by the bipolar-barrel morphology (Fig. 3),
351 however, 5 μ M and 10 μ M IAA increased spindle length and decreased spindle width,
352 indicating that IAA disrupted normal spindle morphology by altering spindle shapes
353 (Fig. 3). In addition, IAA impaired chromosome alignment as evidenced by increased
354 MI-plate width. We also found that 30- μ M-IAA treatment resulted in very long
355 spindles (Fig. S1), which further verified that IAA exposure elongates spindle length
356 in oocytes. It is worth noting that balance between microtubule (MT) polymerization
357 and depolymerization is important for spindle length control and knockdown of some
358 MT depolymerization-related proteins will cause elongated spindles (Do et al., 2014;
359 Wang et al., 2010). Thus, we speculated that IAA (5-30 μ M in our study) might
360 disrupt the MT depolymerization in oocytes, which further study is needed to verify
361 this hypothesis.

362 It is known that spindle assembly checkpoint (SAC) is insufficient for
363 preventing chromosome mis-segregation in mammalian oocytes (Sebestova et al.,
364 2012). Thus, only extreme spindle damage and chromosome misalignment can induce
365 MI arrest to prevent polar body formation. Therefore, we hypothesized that the IAA
366 (5 μ M and 10 μ M)-induced mild abnormality of spindle assembly and chromosome
367 alignment is not sufficient to trigger robust MI arrest. In addition, our data indicated
368 that 30 μ M IAA did cause severe spindle defects and chromosome misalignment (Fig.

369 S1), which suggests that high IAA concentrations might induce MI arrest in oocytes.
 370 Additionally, we observed that the IAA-treated oocytes had more severely misshaped
 371 spindles at MII compared to MI. Therefore, we inferred that a longer IAA exposure
 372 would increase the severity of spindle mis-assembly and chromosome misalignment.
 373 Since abnormal MII spindle is associated with poor pregnancy outcome (Kilani et al.,
 374 2009), IAA also reduced the quality of matured oocytes.

375 Previous studies have associated IAA exposure with oxidative stress (Cemeli
 376 et al., 2006; Wang et al., 2014; Zhou et al., 2015). IAA exposure increased the ROS
 377 levels and induced Nrf2-mediated antioxidant response in HepG2 cells (Wang et al.,
 378 2014). IAA also elevated the ROS levels that cause oxidative stress in primary
 379 cerebellar granule neurons (Zhou et al., 2015) and CHO cells (Cemeli et al., 2006).
 380 Consistent with these previous studies, our data show that IAA increased ROS levels
 381 in mouse oocytes in a dose-dependent manner (Fig. 4). Since excessive ROS
 382 generation will result in oxidative stress that can impede oocyte maturation (Jiao et al.,
 383 2019, 2020), we hypothesized that IAA-induced ROS elevation inhibits oocyte
 384 maturation *in vitro*. Excessive ROS generation is also associated with causing DNA
 385 damage in cells (Salehi et al., 2018; Srinivas et al., 2019). Our results show that IAA
 386 treatment induced a significantly higher level of DNA damage compared to controls
 387 as indicated by γ -H2AX focus formation, a marker for DNA double-strand breaks
 388 (Fig. 5). This result is consistent with other IAA-toxicological studies, such as
 389 research in CHO cells (Plewa et al., 2010), in human peripheral blood lymphocytes
 390 and sperm (Ali et al., 2014), as well as in NIH3T3 cells (Wei et al., 2013).

Collectively, these studies and our data indicate that IAA triggers oxidative stress and DNA damage in different types of cells. In addition, Collins et al. (2015) and Ding et al. (2019) indicated that DNA damage in mouse oocytes can result in MI arrest that can cause PBE failure. We, therefore, suggest that the IAA-induced DNA damage was one of the causes of MI arrest in the IAA-treated oocytes.

Based on our results, we hypothesized that IAA-induced oxidative stress was the main reason for the oocyte-maturation failure, and supplementation of an antioxidant might rescue the maturation rate in IAA-exposed oocytes. NAC is an antioxidant that can decrease the ROS level and alleviate oxidative stress in cells. Using NAC as an antioxidant can reduce toxicant-induced-oxidative stress and rescue PBE failure in porcine and mouse oocytes (Lai et al., 2015; Li and Zhao, 2019). Our results showed that 200 μ M NAC supplementation significantly reduced ROS levels and increased the PBE rate in IAA (5 μ M and 10 μ M)-exposed oocytes (Fig. 7). This indicates that NAC alleviates the meiotic damage caused by IAA and thereby restores oocyte maturation, which directly confirms that IAA-induced oocyte toxicity is mainly mediated by oxidative stress. Notably, IAA-induced damages in HepG2 cells (Wang et al., 2014) and primary cerebellar granule neurons CGNs (Zhou et al., 2015) were also reduced by antioxidants, which further suggests that oxidative stress is the major mediator by which IAA induces defects in a variety of mammalian cells.

5. Conclusion

In conclusion, our data show that IAA exposure impairs meiotic progression and oocyte maturation via disrupting spindle assembly, inducing oxidative stress, and

413 damaging DNA. Moreover, antioxidant NAC supplementation rescues the maturation
414 failure in IAA-exposed oocytes to some extent. This study provides novel information
415 on the toxicological mechanisms underlying the effects of IAA on oocyte maturation
416 and raises concerns of IAA exposure for potential risks of human and animal
417 reproductive health. Although these data suggest that IAA could impair oocyte
418 maturation, further studies are needed to investigate the effects of IAA on oogenesis
419 *in vivo*.

420

421 **CRedit author statement**

422 **Xiaofei Jiao:** Conceptualization, Methodology, Validation, Investigation, Formal
423 analysis, Writing-Original Draft. **Andressa Gonsioroski:** Resources, Writing-Review
424 & Editing. **Jodi A Flaws:** Resources, Writing-Review & Editing, Funding acquisition.
425 **Huanyu Qiao:** Conceptualization, Resources, Project administration, Supervision,
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620 **Figure legend**

621 **Figure 1. IAA exposure disrupted mouse oocyte maturation *in vitro*.** (A)

622 Representative images of oocytes with GVBD (2-h) and PBE (14-h) in the untreated
623 control and IAA-treated groups (5 μ M). The arrows highlight the oocytes with PBE.

624 Scale bar, 100 μ m. (B) The rates of GVBD in the untreated control and IAA-treatment

625 groups (2 μ M, 5 μ M, and 10 μ M) after 2-hour culture. (C) The PBE rates in the

626 untreated control and IAA-treated groups (2 μ M, 5 μ M, and 10 μ M) after 14-hour

627 culture. (D) Representative images of the PBE-failure oocytes selected from the 5-

628 μ M-IAA-treated group that have been cultured for 14 h (left panel), continuing

629 another 10-h culture (right panel). Note: No selected oocytes (n=20) extrude their

630 polar bodies after 24-h culture. Scale bars, 100 μ m. A total of 120 oocytes in the

631 control, 119 oocytes in the 2 μ M group, 120 oocytes in the 5 μ M group, and 120

oocytes in the 10 μ M group were analyzed for GVBD and PBE rate. Data were presented as mean $\pm$ SEM of at least three independent experiments. *** $P < 0.001$, compared with control.

Figure 2. IAA exposure induced metaphase I arrest in mouse oocytes. (A)

Representative images for the different meiotic stages of oocytes from pro-metaphase I (pro-MI) to anaphase/telophase I (A/TI). α -tubulin (green) was immunostained to show spindle morphology. 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) stained chromosomes/DNA (blue). Scale bar, 20 μ m. (B) The proportions of different cell stages (pro-MI, MI, A/TI) in the control, 5 μ M and 10 μ M IAA-treated groups. A total of 66 oocytes in the control, 71 oocytes in the 5 μ M group and 69 oocytes in the 10 μ M group were used for cell cycle analysis. (C) Representative images show the morphologies of oocytes in the IAA (5 μ M)-treated and control groups. Arrows point to the forming polar bodies. Scale bar, 100 μ m. *** $P < 0.001$, compared with control. * $P < 0.05$, 5 μ M versus 10 μ M.

Figure 3. IAA exposure induced abnormalities of spindle assembly and

chromosome alignment in MI mouse oocytes. (A) Representative images of MI spindle morphologies in the control, 5 μ M and 10 μ M IAA-exposed oocytes. Spindle length was calculated by the distance from one spindle pole to the other. Spindle width was represented by the width of microtubules at the MI plate. MI plate width was measured by the axis distance between the two lines at the edges of the DNA. α -tubulin, green; chromosomes/DNA, blue. Scale bar, 10 μ m. (B) Quantitative analysis (mean $\pm$ SEM) of the meiotic spindle length in three treatment groups. (C)

654 Quantitative analysis (mean $\pm$ SEM) of the meiotic spindle width in three treatment
655 groups. (D) Quantitative analysis (mean $\pm$ SEM) of the MI-plate width in three
656 treatment groups. A total of 33 oocytes in the control, 32 oocytes in the 5 μ M group
657 and 33 oocytes in the 10 μ M group were used for spindle length and width analysis; A
658 total of 33 oocytes in the control, 32 oocytes in the 5 μ M group and 32 oocytes in the
659 10 μ M group were used for MI-plate width analysis. $*P < 0.01$, $***P < 0.001$,
660 compared with control. $*P < 0.01$, 5 μ M versus 10 μ M.

661 **Figure 4. IAA exposure increased the ROS level in mouse oocytes.** (A)
662 Representative images of DCHF-DA fluorescence (green) in the control and IAA (5
663 μ M and 10 μ M)-treated oocytes. DIC, differential interference contrast. Scale bar, 100
664 μ m. (B) Quantitative analysis of DCHF-DA fluorescence intensity in the control and
665 IAA (5 μ M and 10 μ M)-exposed groups. A total of 46 oocytes in the control, 47
666 oocytes in the 5 μ M group, and 47 oocytes in the 10 μ M group were used for analysis.
667 Data were presented as mean percentage (mean $\pm$ SEM) of at least three independent
668 experiments. $***P < 0.001$, compared with control. $*P < 0.05$, 5 μ M versus 10 μ M.

669 **Figure 5. IAA exposure induced DNA damage in mouse oocytes.** (A) Oocytes were
670 arrested at the GV stage by IBMX for 8 h with or without IAA exposure, and then
671 these GV-arrested oocytes were collected for DNA damage detection. Representative
672 images of oocytes with DNA double-strand breaks (DSBs) indicated by γ -H2AX foci.
673 Normal oocytes exhibited no or a few foci, whereas more foci can be found in the
674 IAA-exposed oocytes. Green, γ -H2AX; blue, DNA. Scale bar = 5 μ m. (B)
675 Quantification of γ -H2AX foci in each group. A total of 33 oocytes in the control, 36

oocytes in the 5 μ M group, and 37 oocytes in the 10 μ M group were used for analysis. Data were presented as mean percentage (mean $\pm$ SEM) of at least three independent experiments. * P < 0.05, ** P < 0.01, compared with control; * P < 0.05, 5 μ M versus 10 μ M.

Figure 6. IAA exposure disrupted MII spindle formation and chromosome alignment. (A) Representative images of spindle morphology and chromosome alignment in control and IAA-treated MII oocytes. α -tubulin, green; chromosomes/DNA, blue. Scale bar, 20 μ m. Arrows highlight the misaligned chromosomes. (B) The percentage of oocytes with abnormal spindles in the control and 5 μ M IAA-treated groups. (C) The proportion of oocytes with misaligned chromosomes in the control and 5 μ M IAA-treated groups. A total of 54 MII oocytes in the control and 54 in the 5 μ M IAA-treated group were used for analysis. Data were presented as mean percentage (mean $\pm$ SEM) of at least three independent experiments. ** P < 0.01, compared with control.

Figure 7. NAC rescued the meiotic arrest in IAA-exposed mouse oocytes by reducing oxidative stress. (A) Representative images of ROS levels in the control, IAA-treated, and NAC+IAA-treated oocytes. Scale bar, 100 μ m. (B) Fluorescence intensity of ROS in the oocytes from different treatment groups. A total of 31 oocytes in the control, 32 oocytes in the control + NAC, 33 oocytes in the 5 μ M group, 32 oocytes in the 5 μ M + NAC group, 32 oocytes in the 10 μ M group, and 32 oocytes in the 10 μ M + NAC group were used for ROS analysis. (C) Representative images of oocytes with PBE (14-h) in the control, 5 μ M, and 10 μ M IAA-treated groups with

698 and without NAC treatment. The arrows highlight the oocytes with PBE, Scale bar,
699 100 μm . (D) The PBE rates in the control, 5 μM , and 10 μM IAA-treated groups with
700 and without NAC treatment after 14-hour culture. A total of 65 oocytes in the control,
701 59 oocytes in the control + NAC, 73 oocytes in the 5 μM group, 73 oocytes in the 5
702 μM + NAC group, 69 oocytes in the 10 μM group and 69 oocytes in the 10 μM +
703 NAC group were used for PBE analysis. Data were presented as mean percentage
704 (mean $\pm$ SEM) of at least three independent experiments. $**P < 0.01$, $***P < 0.001$; 5
705 μM versus 5 μM + NAC; 10 μM versus 10 μM + NAC.

Figures

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