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JQ-1 ameliorates schistosomiasis liver granuloma in mice by suppressing male and female reproductive systems and egg development of *Schistosoma japonicum*

Short title: JQ-1 ameliorates schistosomiasis liver granuloma in mice

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Abstract

Schistosomiasis is a serious and widespread parasitic disease caused by infection with *Schistosoma japonicum*. Because the parasite's eggs are primarily responsible for schistosomiasis dissemination and pathogenesis, inhibiting egg production is a potential approach to control the spread and severity of the disease. The bromodomain and extra-terminal (BET) proteins represent promising targets for the development of epigenetic drugs against Schistosoma. JQ-1 is a selective inhibitor of the BET protein family. In the present study, JQ-1 was applied *S. japonicum* in vitro. By using laser confocal

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22 scanning microscopy and EdU incorporation assays, we showed that application of JQ-
23 1 to worms in vitro affected egg laying and the development of both the male and female
24 reproductive systems. JQ-1 also inhibited the expression of the reproductive-related
25 genes *SjPlk1* and *SjNanos1* in *S. japonicum*. Mice infected with *S. japonicum* were
26 treated with JQ-1 during egg granuloma formation. JQ-1 treatment significantly
27 reduced the size of the liver granulomas and levels of serum alanine aminotransferase
28 and aspartate aminotransferase in mice and suppressed both egg laying and the
29 development of male and female *S. japonicum* reproductive systems in vivo. Moreover,
30 the mRNA expression levels of some proinflammatory cytokines were decreased in the
31 parasites. Our findings suggest that JQ-1 treatment attenuates *S. japonicum* egg–
32 induced hepatic granuloma due at least in part to suppressing the development of the
33 reproductive system and egg production of *S. japonicum*. These findings further suggest
34 that JQ-1 or other BET inhibitors warrant additional study as a new approach for the
35 treatment or prevention of schistosomiasis.

36 Keywords: Hepatic granuloma; Reproductive systems; JQ-1; *Schistosoma japonicum*.

37 Author summary

38 Among neglected tropical diseases, schistosomiasis is a serious disease caused by
39 infection with the parasite *Schistosomiasis japonicum*. Treatment of schistosomiasis is
40 currently almost exclusively with praziquantel, which kills mainly adult parasites, with
41 minimal effectiveness against immature schistosomes and eggs. However, the
42 parasite's eggs are primarily responsible for schistosomiasis dissemination and
43 pathology. In addition, overuse of praziquantel in epidemic areas has led to drug

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44 resistance and a reduced cure rate. Thus, new parasite targets for the development of
45 novel therapeutics are crucial. Here, we evaluated the potential of JQ-1, a bromodomain
46 and extra-terminal protein inhibitor, to suppress the production of *S. japonicum* eggs.
47 Application of JQ-1 to *S. japonicum* in vitro decreased the number of mature germ cells,
48 the rates of oviposition, and the number of eggs produced in each male-female pairing.
49 JQ-1 treatment of mice infected with *S. japonicum* ameliorated hepatic granuloma and
50 decreased serum liver enzymes, suggesting improved liver function. These results
51 indicate that JQ-1 inhibits reproductive development and egg production in *S.*
52 *japonicum*, providing supporting evidence that JQ-1 warrants additional study for use
53 as a novel approach in the prevention or treatment of schistosomiasis.

54 Introduction

55 Schistosomiasis is an acute and chronic parasitic disease caused by infection with
56 Schistosoma, a parasite that is endemic in 78 countries and is responsible for
57 approximately 280,000 deaths each year [1]. In China, zoonotic schistosomiasis caused
58 by S. japonicum is major public health threat affecting more than a million people and
59 hundreds of thousands of livestock in China [2].

60 Praziquantel is a widely used, high-efficiency, broad-spectrum, oral antiparasitic
61 drug for the treatment of various forms of schistosomiasis, but praziquantel kills only
62 adult worms and is minimally effective against immature schistosomes and eggs [2-3]
63 In addition, the repeated and large-scale use of praziquantel in epidemic areas has led
64 to drug resistance and a reduced cure rate [4-5]. Thus, there is an urgent need to identify

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65 new targets for the development of novel parasitic therapeutics. Owing to the key role
66 of fertilized eggs in maintaining the life cycle and inducing pathogenesis [2-3], blocking
67 egg production is a potential alternative approach to control the occurrence,
68 development, and spread of schistosomiasis.

69 The bromodomain and extra-terminal (BET) family of proteins specifically
70 recognizes acetylated lysine residue sites and participates in the regulation of epigenetic
71 protein expression, which plays a key role in regulating various biological processes
72 [6]. JQ-1 is a selective inhibitor of BET family proteins and has been shown to have
73 promising anti-tumor and anti-inflammatory effects [7]. In a pilot study, we used JQ-1
74 to treat hepatic granuloma caused by infection with *Schistosoma japonicum*. Mice
75 infected with S. *japonicum* cercariae were injected intraperitoneally with JQ-1 (50
76 mg/kg) during egg granuloma formation. Unexpectedly, JQ-1 significantly reduced the
77 size of the liver granuloma and the egg burden; however, JQ-1 treatment had no effect
78 on worm load. We hypothesized that JQ-1 would be effective in inhibiting egg
79 production in S. *japonicum* and sought to learn the mechanisms underlying this effect.

80 Thus, the aim of the present study was to confirm that JQ-1 reduces egg production
81 of *S. japonicum* and to investigate the potential mechanisms undergirding this effect.
82 To that end, we applied JQ-1 to schistosomes in vitro and assessed the effects on their
83 reproductive development and egg production. We also treated C57BL/6 mice infected
84 with *S. japonicum* with JQ-1 to assess the effects of the drug on hepatic granuloma and
85 liver function. Our findings indicated that JQ-1 inhibited the reproductive development
86 of males and females and egg production in *S. japonicum* and ameliorated hepatic

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87 granuloma and improved liver function in infected mice. These findings lay a
88 foundation for further study to develop JQ-1 or other BET inhibitors as a new approach
89 for the treatment and prevention of schistosomiasis.

90 Materials and Methods

91 Animals and parasites

92 Female Kunming mice (6-8 weeks old) and female C57BL/6 mice (6-8 weeks old) were
93 provided by the Experimental Animal Center of Anhui Province in Hefei, China. The
94 mice were housed under specific pathogen-free conditions at Anhui Medical University.
95 *Oncomelania hupensis* snails infected with *S. japonicum* (a Chinese mainland strain)
96 were purchased from the Jiangxi Provincial Institute of Parasitic Diseases in China. All
97 experiments carried out on animals were conducted in accordance with and were
98 approved by the Animal Ethics Committee of Anhui Medical University (approval No.
99 LLSC20170247).

100 Treatment of schistosomes with JQ-1 in vitro

101 Cercariae were shed in a beaker after exposing 30 *O. hupensis* infected with *S.*
102 *japonicum* to sunlight for 4 h (25–28 °C). For mixed infections, cercariae released from
103 several infected *O. hupensis* were used. Kunming mice were infected percutaneously
104 with 80–90 cercariae and were humanely killed on the 28th day after infection. All
105 paired parasites were harvested by perfusion and washed three times with RPMI-1640
106 medium. The worms were then cultured in vitro with RPMI-1640 (Gibco, Grand Island,

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107 NY, USA) at 37 °C and 5% CO2. The RPMI-1640 medium was supplemented with
108 10,000 U/mL penicillin, 10 mg/mL streptomycin, 250 μg/mL amphotericin B (Sangon
109 Biotech, Shanghai, China), 15% fetal calf serum (Gibco), and glutamine (Gibco). For
110 each experiment, 15 pairs of *S. japonicum* were maintained in a 6-well plate (i.e., 15
111 pairs/well). JQ-1 (Cat. No. HY-13030, MedChem Express; USA), was dissolved in
112 dimethyl sulfoxide (DMSO). In each experimental group, 15 paired parasites were
113 incubated in 3 mL of medium and treated with different concentrations of JQ-1 (0 μM,
114 5 μM, 10 μM, and 15 μM). All parasites were cultured at 37 °C for 9 d, and culture
115 media was changed every 24 h. During this time, the viability and morphology of
116 parasites, worm pairings, and the number of eggs were observed and recorded.

117 Confocal laser scanning microscopy (CLSM)

118 For morphological analysis, collected worms were fixed in a solution of alcohol (95%),
119 formalin (3%), and glacial acetic acid (2%)) for at least 24 h. Worms were stained in
120 hydrochloric acid–carmine dye (Ourchem, Shanghai, China) for 17 h and then destained
121 in acidic 70% ethanol until the worms turned light pink. The worms were dehydrated
122 in a graded ethanol series (70%, 90%, and 100%), cleared in 50% xylene diluted in
123 ethanol and 100% xylene for 1 min each, mounted onto slides with neutral gum, sealed
124 with cover glass, and laid flat to dry. The morphology of their reproductive organs was
125 observed with a CLSM (ZEISS LSM 880, Germany) using an emission wavelength of
126 488 nm. Images were captured and stored at 1024 × 1024 pixels.

127 5-ethynyl-2′-deoxyuridine (EdU)-incorporation assay

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128 For EdU labelling and detection of proliferating cells, paired worms treated with JQ-1
129 and control worms were incubated with 10 mM of EdU in medium for 24 h.
130 BeyoClick™ EdU-594 Cell Proliferation Kits (Beyotime, Shanghai, China) were used
131 to detect EdU incorporation. Couples were separated, fixed, and stained as described
132 above, with minor alterations. The couples were rinsed twice in PBS and stained with
133 Hoechst 33342 (diluted 1:1000 in PBS) in the dark for 10 min at room temperature. The
134 worms were examined by CLSM using a ZEISS LSM 880 confocal microscope at a
135 wavelength of 405 nm (for Hoechst) and 543 nm (for Azide 594).

136 Treatment of schistosomes with JQ-1 in vivo

137 Four weeks after mice were infected with *S. japonicum,* mice in the experimental group
138 were injected intraperitoneally with JQ-1 (50 mg/kg body weight per day), and mice in
139 the control group were injected intraperitoneally with vehicle, namely, (2-
140 hydroxypropyl)-β-cyclodextrin (HP-β-CD; Cat. No. 778966, Sigma; USA) 10%
141 (wt/vol), once daily for 15 d. Animals were humanely killed 24 h after the last
142 administration. The parasites, serum, and liver from each mouse were collected for
143 subsequent experimental analyses.

144 Quantitative PCR

145 Total RNA from adult *S. japonicum* worms or the liver of each mouse was isolated
146 using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA). The total RNA
147 concentration and purity were detected using a NanoDrop 2000 (Thermo Fisher

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148 Scientific, USA). Total RNA (500 ng) from the worms was reverse transcribed into
149 cDNA by using a PrimeScript RT Reagent Kit (TaKaRa, Dalian, China) according to
150 the manufacturer's instructions. A reliable reference gene for transcriptomic analysis
151 of *S. japonicum*, *PSMD4*, was used as a control gene in the assays [8] and GAPDH was
152 used as a control gene for transcriptomic analysis of the liver. The experiment was
153 carried out using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster
154 City, CA, USA). The relative expression level of each gene was analyzed using SDS
155 v.1.4 software (Applied Biosystems). The procedure for quantitative PCR was
156 conducted as described previously [9], and the primers were designed and synthesized
157 by Sangon Biotech Co. Ltd. The PCR primer sequences are described in the
158 Supplementary Material.

159 Serum liver enzyme quantification

160 For assessment of mouse liver function, a serum aminotransferase test kit (Nanjing
161 Jiancheng Bioengineering Institute, Nanjing, China) was used to measure the levels of
162 serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST),
163 according to the manufacturer's instructions. The levels of serum ALT and AST are
164 reported in units per liter.

165 Egg count in liver tissue

166 Approximately 0.1 g of liver tissue was taken from each mouse and weighed. Potassium
167 hydroxide (10%; 1 mL) was added to the liver tissue for digestion at 37 °C for 2 h. The
168 number of eggs in each sample was then counted using a light microscope.

169 Histology and immunohistochemistry of the liver sections

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170 Fresh liver tissue (1.0 g) was fixed in 1% buffered formalin and embedded in paraffin.
171 The deparaffinized tissue sections were affixed to slides, and sections (thickness, 4 μm)
172 were stained with hematoxylin and eosin and examined for quantitative and qualitative
173 changes. Computer-assisted morphometric software ((Image-Pro Plus; Media
174 Cybernetics) was used to determine the total areas of the tissue and granuloma on each
175 slide so that the area of the granulomas could be reported as a percentage of the total
176 area for each slide. For each specimen, at least three non-continuous slides were
177 measured, and the mean values obtained from eight mice from each group were used
178 for statistical analysis.

179 Statistical analysis

180 Statistical analysis was performed using GraphPad Prism software (version 6.0). All
181 data were obtained from three independent experiments, each using triplicate samples
182 and following the same protocol. The statistical significance of the difference between
183 two data sets was analyzed using Student's t-test, and one-way analysis of variance
184 (ANOVA) was used for multiple comparisons, followed by Tukey's post hoc tests when
185 appropriate. Data are presented as means ± SEM and were considered statistically
186 significant for P-values < 0.05.

187 Results

188 Effects of JQ-1 treatment on pairing rate and egg production

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189 The number of male-female paired worms was counted on the 10th day of culture to
190 determine the effect of JQ-1 treatment on the pairing rate. We found that the number of
191 paired worms in the cultures treated with JQ-1 was similar to that in the control group
192 treated with vehicle (Fig 1A). No significant changes in schistosome activity or in the
193 number of viable worms were detected between the JQ-1–treated group and the control
194 group. However, the number of eggs collected in the medium and counted using light
195 microscopy was decreased in the cultures treated with JQ-1 compared with controls
196 (Fig 1 C–F). To further analyze the effects of JQ-1 on egg production in the paired
197 females, we counted egg numbers and found that compared with the DMSO-treated
198 group, the number of eggs ($P < 0.05$) in the JQ-1–treated group decreased in a
199 concentration-dependent manner (Fig 1B).

200 **Fig 1. Effect of JQ-1 on male-female pairing rate, egg production, and egg**
201 **morphology in *S. japonicum*.** Effects of different concentrations (5 μM, 10 μM, and
202 15 μM) of JQ-1 application on male-female pairing stability (A), egg production (B),
203 and egg morphology (C–F) in *S. japonicum* pairs cultured in vitro for 10 days. Data
204 represent the mean ± SEM of three independent experiments. Scale bars: 200 μm.
205 Asterisks show statistical differences (***$P < 0.001$) tested by one-way ANOVA with
206 multiple comparisons (Tukey's post-hoc test).

207 JQ-1 treatment decreases mitotic activity in somatic and
208 germ cells

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209 We investigated whether JQ-1 affects mitosis in *S. japonicum* by performing EdU-
210 incorporation assays using JQ-1–treated worms to assess cell proliferation. Worm pairs
211 treated with JQ-1 for 10 d exhibited a substantial decrease in the number of EdU-labeled
212 cells in the gonads, parenchyma, and subtegument of both sexes. In the untreated
213 control group, a substantial number of EdU-labeled cells were detected in the
214 vitellarium and ovary of adult females as well as in the testis and parenchyma of adult
215 males (Fig 2), which indicated high mitotic activity in these organs. Adult worms
216 treated with JQ-1 for 9 d showed a slight decrease in the number of EdU-positive cells
217 in the vitellarium of the females and the testis and parenchyma of the males; greater
218 decreases were observed with increasing concentrations of JQ-1. At the highest
219 concentration, JQ-1–treated worm organs and tissues had almost no EdU-labeled cells
220 (Fig 2D, H).

221 **Fig 2. Effect of JQ-1 on cell proliferation in male-female pairs of *S. japonicum*.** Red
222 signals indicate active mitotic cells labeled by EdU; blue signal, Hoechst-positive cells.
223 (A–D) Male *S. japonicum* and (E–H) female *S. japonicum*. EdU-incorporated cells are
224 detected in the testes and parenchyma of untreated males (A) and in the vitellarium and
225 ovary of untreated females (E). EdU-positive cells are detected after application of JQ-1
226 at 5 μM (B, F), 10 μM (C, G), and 15 μM (D, H). Scale bars: 200 μm.

227 Effects of JQ-1 treatment on reproductive organ development

228 Consistent with the observed decreased egg production, CLSM analyses of worm pairs
229 treated with JQ-1 revealed morphologic abnormalities in the gonads of both sexes. After

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230 treatment for 10 d, the length and width of the ovaries in females treated with JQ-1 were
231 significantly smaller than those of untreated controls (Fig 3I–K). In the control group,
232 no morphological anomalies were observed in the testes of the males (Fig 4A, E) or the
233 ovaries of the females (Fig 3A, E). Furthermore, the vitellaria of control females
234 contained differentiating vitellocytes. The ovaries of the DMSO-treated female
235 schistosomes were composed of small immature oocytes in the anterior part and larger
236 primary oocytes in the posterior part. The results of CLSM (Fig 4E–H) showed that the
237 number of spermatozoa in the seminal vesicles of schistosomes in the JQ-1–treated
238 group was reduced and the development of the spermatozoa was impaired. The testes
239 of DMSO-treated male schistosomes were composed of several testicular lobes
240 arranged bead-like, and each testicular lobe contained a large number of spermatocytes
241 and spermatogonia at different stages. In the group treated with JQ-1, the morphology
242 of whole germ cells in both the testis and ovary were markedly changed. Those changes
243 were more obvious with increasing concentrations of JQ-1. In the ovaries, the sizes of
244 the primary oocytes and immature oocytes were reduced, and the cells of the JQ-1–
245 treated groups were not as full as the cells of DMSO-treated groups (Fig 3A–D). The
246 size of the testicular lobes in the group treated with the high concentration of JQ-1 was
247 much smaller than that in the DMSO-treated group, and the numbers of spermatogonia
248 and spermatocytes in the male testes were significantly reduced and more loosely
249 arranged (Fig 4D). Large pore-like structures were observed in the testes and ovaries of
250 males and females, respectively (Figs 3 and 4, arrows). These morphological changes
251 in both females and males were greatest after treatment with the highest concentration

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252 (15 µM) of JQ-1. Compared with controls, the group with JQ-1 treatment showed a
253 markedly reduced diameter of the testicular lobes (Fig 4I), which was paralleled by a
254 reduction in cell density within the testes as well as by empty seminal vesicles.

255 **Fig 3. Morphological changes of ovaries and yolk glands in female *S. japonicum***
256 **treated with JQ-1.** Worms were stained with carmine hydrochloride and analyzed
257 using confocal laser scanning microscopy. (A, E) Control worms; worms treated with
258 JQ-1 at 5 µM (B, F), 10 µM (C, G), and 15 µM (D, H). Arrows indicate large pore-like
259 structures. Abbreviations: ov, ovary; v, vitellarium; (A–H) Scale bars: 20 µm.
260 Comparison of the length, width, and area of the ovary after JQ-1 application at the
261 indicated concentration (I–K) for 10 d. Data represent the mean ± SEM (n ≥ 15 for each
262 group). Asterisks show statistical differences (**$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)
263 tested by one-way ANOVA with multiple comparisons.

264 **Fig 4. Morphological changes of spermatozoa in testes and seminal vesicles of *S.***
265 ***japonicum* treated with JQ-1 in vitro.** Worms were stained with carmine
266 hydrochloride and analyzed using confocal laser scanning microscopy. (A, E) Control
267 worms; worms treated with JQ-1 at 5 µM (B, F), 10 µM (C, G), and 15 µM (D, H).
268 Arrows indicate large pore-like structures. (A–D) Scale bars: 20 µm; (E-H) Scale bars:
269 10 µm. Abbreviations: t, testis; SV, sperm vesicle. (I) Comparison of the diameter of
270 the testicular lobes after JQ-1 application at the indicated concentration for 10 d. Data
271 represent the mean ± SEM (n ≥ 15 for each group). Asterisks show statistical

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272 differences (**$P < 0.01$; ***$P < 0.001$) tested by one-way ANOVA with multiple
273 comparisons.

274 JQ-1 treatment decreases *SjNanos1*, *SjPlk1* mRNA levels

275 To explore the mechanisms undergirding the observed effects of JQ-1 on *S. japonicum*,
276 we used quantitative PCR to detect the levels of the *S. japonicum* protein coding genes
277 polo-like kinase 1 (*SjPlk1*) and *SjNanos1*, two genes related to schistosome
278 reproduction, after application of different concentrations of JQ-1 in vitro. Compared
279 with the control group, the expression levels of *SjPlk1* mRNA in the JQ-1–treated
280 worms were down-regulated in both males (Fig 5A) and females (Fig 5B), and this
281 effect was more marked with increasing concentrations of JQ-1. Similarly, the
282 expression levels of *SjNanos1* mRNA were also down-regulated in both males and
283 females, and this effect was also more marked with increasing concentrations of JQ-1.

284 **Fig 5. Results of quantitative PCR analyses of *S. japonicum* cultured with or**
285 **without JQ-1 for 10 days.** Relative transcription level of *Nanos1* in male (A) and
286 female *S. japonicum* (B). Relative transcription level of *Plk1* in male (C) and female
287 (D) *S. japonicum*. Data represent the mean ± SEM of three independent experiments.
288 Asterisks show statistical differences (*$P < 0.05$; **$P < 0.01$) tested by one-way
289 ANOVA with multiple comparisons.

290 JQ-1 ameliorates liver granuloma caused by *S. japonicum*
291 infection

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292 In the fourth week after *S. japonicum* infection, mice in the experimental group were
293 injected with JQ-1, and mice in the control group were injected with the vehicle HP-β-
294 CD, once daily for 15 d. All mice were humanely killed after 15 d of treatment (Fig
295 6A). As shown in Fig 6B, livers obtained from mice in the HP-β-CD group had large
296 agglomeration, and granuloma inflammation was severe. However, there was marked
297 reduction of liver surface granulomatous nodules in the JQ-1–treated group. The livers
298 obtained from mice in the JQ-1-treated group were lighter and more vivid in color, and
299 the surface was relatively smooth compared with the livers from mice in the control
300 group. Hematoxylin and eosin staining of the liver showed that the percentage of the
301 area of the liver that had granulomas in the JQ-1–treated group was significantly
302 reduced compared with that in the HP-β-CD control group (Fig 6C) ($P < 0.05$). In
303 addition, the weights of the liver and spleen obtained from mice treated with JQ-1 were
304 significantly lower than those from control mice (Fig 6D). Moreover, the results of the
305 AST and ALT assays showed that the activity of serum transaminase in the JQ-1–
306 treated group was significantly lower than that in the control group (Fig 6F) ($P < 0.05$).

307 **Fig 6. Effect of JQ-1 treatment on liver granuloma in mice infected with *S.***
308 ***japonicum*.** (A) Protocol used to assess liver granuloma in mice. (B) Gross appearance
309 of livers obtained from mice infected with *S. japonicum* and treated with JQ-1 or vehicle
310 (HP-β-CD). Liver slices stained with hematoxylin and eosin. Scale bars: 500 μm. (C)
311 Measurement of granuloma area as a percentage of total area as assessed by computer-
312 aided morphometry. (D) Liver weight of *S. japonicum*–infected mice treated with JQ-
313 1 or HP-β-CD. (E) Spleen weight of *S. japonicum*–infected mice treated with JQ-1 or

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314 HP-β-CD. (F) Effect of JQ-1 treatment on serum alanine aminotransferase (ALT) and
315 aspartate aminotransferase (AST) in mice infected with *S. japonicum*. Data represent
316 the mean ± SEM (n = 9 for each group). Asterisks denote statistically significant
317 differences (Student's t-test, **P* < 0.05; ****P* < 0.001) vs. the HP-β-CD–treated control
318 group. ns, not significant.

319 To further explore the effect of JQ-1 treatment to ameliorate hepatic granuloma in mice
320 infected with *S. japonicum*, we used quantitative PCR to detect the expression levels of
321 a series of inflammatory factors. The mRNA expression levels of the genes in the HP-
322 β-CD–treated control group were set at 1. As shown in Fig 7, the mRNA expression
323 levels of the inflammatory factors in the JQ-1–treated group relative to those in the
324 control group were significantly decreased (*P* < 0.05). Notably, the expression level of
325 interleukin 13 (IL-13), an inflammatory factor closely related to the formation of
326 granuloma caused by *S. japonicum*, was significantly reduced.

327 **Fig 7. Effect of JQ-1 treatment on mRNA expression of inflammatory-related**
328 **genes in the liver of mice infected with *S. japonicum*.** The mRNA levels are expressed
329 relative to those in controls following normalization with GAPDH. Data represent the
330 mean ± SEM (n = 9 for each group). Asterisks denote statistically significant
331 differences (Student's t test, **P* < 0.05).

332 Effects of JQ-1 treatment on schistosome eggs in the liver and 333 on adult worms in mice infected with *S. japonicum*

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334 The above results suggested that JQ-1 alleviated liver injury caused by schistosome
335 infection to some extent and reduced the formation of hepatic granuloma in mice. To
336 observe whether JQ-1 affected *S. japonicum* eggs in the liver, we evaluated the quantity
337 of eggs in the liver of mice in the JQ-1–treated group compared with that in the HP-β-
338 CD–treated control group after schistosome infection. The liver tissue obtained
339 following digestion with 10% potassium hydroxide was used to observe the
340 morphology of the eggs and to count them. We found that the proportion of abnormally
341 small or dead eggs was increased in the JQ-1–treated group (Fig 8). The volume of eggs
342 in the liver of JQ-1–treated infected mice was approximately 40% lower than that of
343 control mice injected with HP-β-CD. By contrast, the numbers of adult worms and
344 worm pairs in the livers of the JQ-1–treated group were not affected. The percentage of
345 the liver that was granuloma tissue in the JQ-1–treated group was significantly
346 decreased compared with control. Although this effect in the treated group may have
347 been due to the significant decrease in the number of eggs or to the increase in the
348 number of small or dead eggs, it may also be related to the immune regulation of JQ-1
349 in mice. We used an EdU-incorporation assay to assess the proliferation of germ cells
350 in schistosomes of infected mice (Fig 8). Although some differences between the
351 control group and the treated group were observed, the differences were not as obvious
352 as those observed in the in vitro experiments.

353 **Fig 8. JQ-1 treatment alters germ cell proliferation of *S. japonicum* and egg**
354 **production in the liver of mice infected with *S. japonicum*.** (A) Egg morphology and
355 (B) production in the liver. (C) Numbers of adult worms and (D) worm pairs in the

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356 liver. Red signals indicate active mitotic cells labeled by EdU; blue, Hoechst-positive
357 cells. EdU-incorporated cells in control worms were detected in the testes and
358 parenchyma of males (E) and in the vitellarium and ovary of females (F). (G, H) EdU-
359 positive cells detected in *S. japonicum* of mice treated with JQ-1. (A) Scale bars: 500
360 µm. (E-H) Scale bars: 100 µm. Data represent the mean ± SEM (n = 9 for each group).
361 Asterisks denote statistically significant differences (Student's t test, ** $P < 0.01$).

362 CLSM analyses of the JQ-1–treated group revealed morphologic abnormalities in the
363 gonads of both sexes. In the control HP-β-CD–treated group, no morphological
364 anomalies were observed in the ovaries of the females (Fig 9A) or the testes of the
365 males (Fig 10B). By contrast, compared with the control group, the number of
366 spermatozoa in the seminal vesicles of schistosomes in the JQ-1–treated group was
367 reduced and the development of spermatozoa was impaired (Fig 9C, F). In addition, the
368 overall morphology of the germ cells of schistosomes in both the testis and ovary were
369 markedly changed. The sizes of the primary oocytes and immature oocytes were
370 reduced, and the cells in the JQ-1–treated group were not as filled as the cells in the
371 HP-β-CD group (Fig 9A, D). Moreover, large pore-like structures could be found in the
372 testes and ovaries of male and female schistosomes, respectively, in the JQ-1–treated
373 group (Fig 10, arrows).

374 **Fig 9. Morphological changes in the testis and ovary of *S. japonicum* treated with**
375 **JQ-1 in vivo.** Worms were stained with carmine hydrochloride and analyzed using
376 confocal laser scanning microscopy. (A–C) Testes and seminal vesicles of worms in

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377 control mice. (D–F) Testes and seminal vesicles of worms in mice treated with JQ-1.
378 Abbreviations: ov, ovary; t, testes; SV, seminal vesicles. Scale bars: 20 μm.

379 Discussion

380 The present study assessed the effects of JQ-1 application on *S. japonicum* in vitro
381 and in vivo and investigated the potential mechanisms undergirding the observed
382 effects. The results of our in vitro studies indicated that although JQ-1 application did
383 not affect the number or pairing of adult schistosomes, the number of eggs decreased
384 in a concentration-dependent manner. In addition, mitotic activity in the somatic and
385 germ cells of the adult worms decreased. The numbers of spermatogonia and
386 spermatocytes were significantly decreased and the testicular lobes were significantly
387 smaller in male schistosomes treated with JQ-1 compared with schistosomes in the
388 control group. Moreover, large pore-like structures were observed in the testes and
389 ovaries of JQ-1–treated schistosomes. These results suggested that JQ-1 specifically
390 inhibited the proliferation of germ cells. Our EdU incorporation assays confirmed that
391 JQ-1 reduced the number of proliferating cells in both the ovaries and testes of
392 schistosomes. Proliferation of those cells is essential for the initiation and continuous
393 production of mature germ cells. Treatment with JQ-1 also decreased the expression
394 levels of two genes related to schistosome reproduction, *SjPlk1* and *SjNanos1*, in a
395 concentration-dependent manner. Thus, this study is the first, to our knowledge, to
396 show that JQ-1 is effective against reproductive development and egg production of
397 adult *S. japonicum* in vitro. In schistosomiasis in humans, morbidity is mainly attributed
398 to the eggs because of the granulomatous inflammatory reaction caused by the host

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399 immune response to egg antigens [2-3]. Thus, we assessed the ability of JQ-1 to treat
400 hepatic granuloma in mice infected with *S. japonicum* in vivo. JQ-1 treatment
401 significantly decreased the percentage of the area of the liver with granulomas, the
402 activity of liver serum transaminase, and schistosome egg production in the liver of
403 mice without affecting the survival of adult worms. The attenuated egg production was
404 accompanied by decreased expression levels of proinflammatory cytokines, which may
405 have contributed to the amelioration of hepatic granuloma. Taken together, our findings
406 provide evidence supporting the development of JQ-1 as an anti-schistosomal agent.
407 The BET family proteins are characterized by the presence of two tandem
408 bromodomains and an extra-terminal domain, which are found in BRD2, BRD3, BRD4,
409 and BRDT in mammalians [6] The domain organization of mammalian BET proteins
410 is conserved in orthologs, including in Drosophila FSH and *Saccharomyces cerevisiae*
411 Bdf1 and Bdf2. Bromodomains that specifically bind acetylated lysine residues in
412 histones serve as chromatin-targeting modules that decipher the histone acetylation
413 code. BET proteins play a crucial role in regulating gene transcription through
414 epigenetic interactions between bromodomains and acetylated histones during cell
415 proliferation and differentiation [10-11]. *Brd2* mRNA is express in distinct patterns
416 during ovarian folliculogenesis, which is essential for embryonic development in the
417 mouse [12-13], Brdt acetylated histone H4-dependent chromatin remodeling in
418 mammalian spermiogenesis is essential for male germ cell differentiation [14-15]. In
419 addition, a BRDT-like function in Drosophila plays crucial roles in spermatid
420 differentiation [16]. Epigenetic modifications, including DNA methylation, histone

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421 modifications, and non-coding RNAs, play important roles in the development and
422 reproduction of schistosomes [17]. SmGCN5 and SmCBP1are two histone
423 acetyltransferases of *S. mansio*n, the knockdown of SmGCN5 or SmCBP1 significantly
424 inhibited Smp14 expression, which compromised the reproductive system of mature
425 females, egg-laying and egg morphology [18]. Sirtuins are a family of histone
426 deacetylases, and sirtuin inhibitors can inhibit apoptosis and death in schistosome
427 larvae, the disruption of adult worm pairs, inhibition of egg laying and damage to the
428 male and female worm reproductive systems [19-20].
429 As a first-in-class potent and selective inhibitor of the BET signaling pathway, JQ-1
430 has been widely used in biology studies. The results of some of those many studies
431 indicate that JQ-1 interacts with the BRD pocket in a manner competitive with
432 acetylated peptide binding, resulting in the displacement of BET proteins from
433 acetylated chromatin in cells exposed to these inhibitors along with their associated
434 transcript initiation and elongation factors. JQ-1 has also been used as a
435 pharmacological tool for elucidating the roles and functions of BET in mammals.
436 However, little is known about the effect of JQ-1 on parasites.
437 Nanos has been described as a necessary factor in the differentiation and migration
438 of primordial germ cells, which play an essential role in the proliferation of germ cells
439 in schistosomes [21-22]. SmPlk1 regulates the cell cycle G2/M transition in *Xenopus*
440 oocytes, which is important for cell-cycle progression in the gonadal cells of
441 Schistosoma [23-24]. In the present study, we investigated whether JQ-1 also affected
442 the transcript level of *Nanos1* and *Plk1*. Indeed, treatment with JQ-1 significantly

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443 reduced the transcript level of both these genes in male and female worms, which likely
444 affected the proliferation of the gonadal cells in Schistosoma.

445 This study has limitations that should be considered when interpreting our results.
446 On the basis of previous publications [25-26], we used only a single dose of JQ-1 (50
447 mg/kg) to treat mice infected with *S. japonicum* for 15 days. Thus, we were unable to
448 make any comparisons of the effects after various treatment times or dosage on
449 parasites in infected mice. Future studies are needed to find the therapeutic optimum
450 dosage.

451 In conclusion, our data showed that JQ-1 treatment ameliorated *S. japonicum* egg–
452 induced hepatic granuloma, which may be due in part to suppressing the development
453 of both the male and female reproductive systems and female egg production in this
454 parasite. Our findings provide theoretical and practical evidence supporting the
455 development of JQ-1 as an anti-schistosomal agent.

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559

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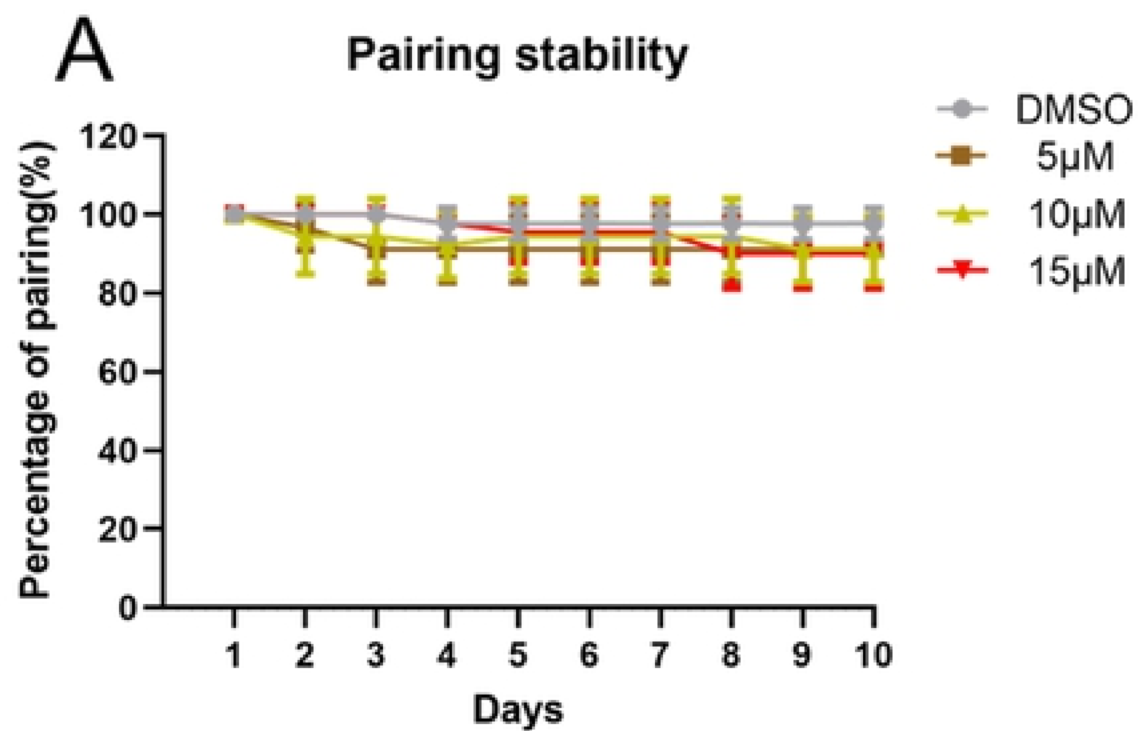
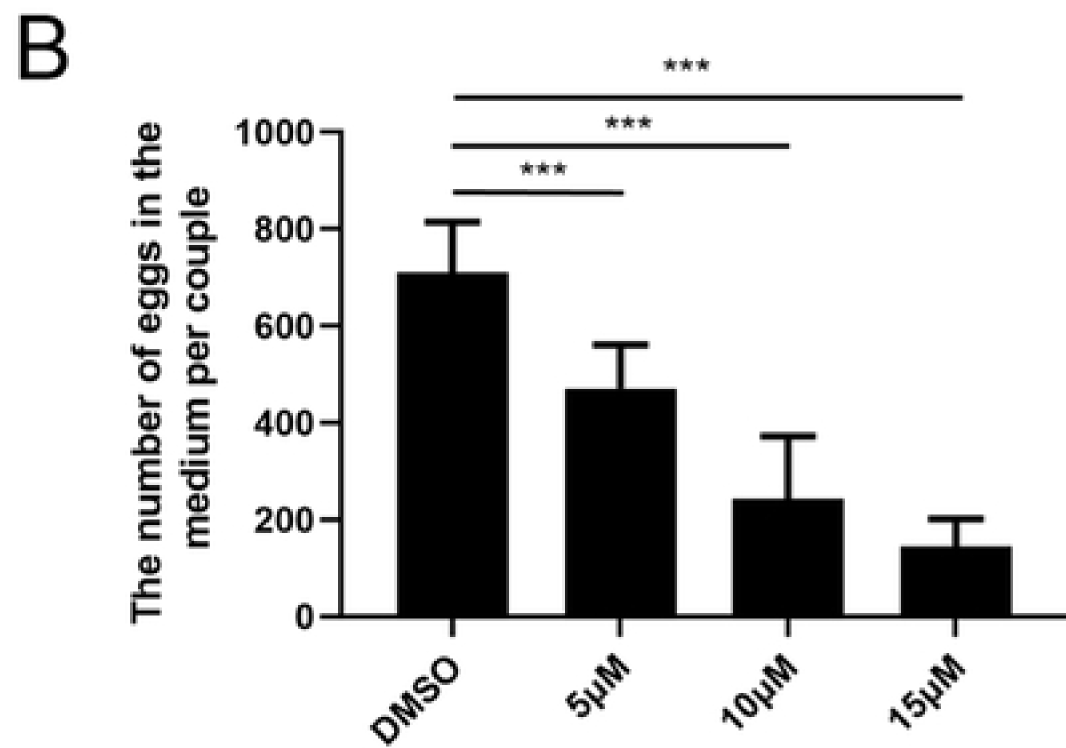
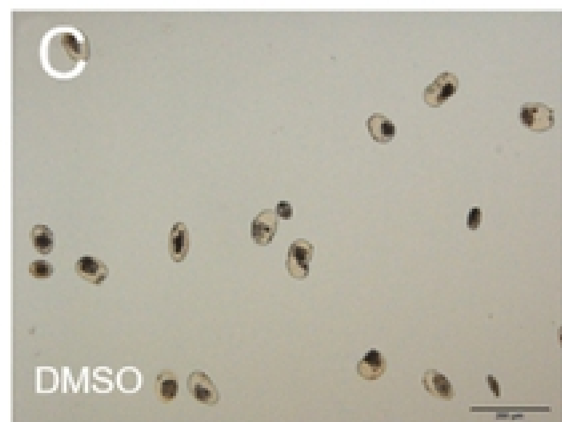
566

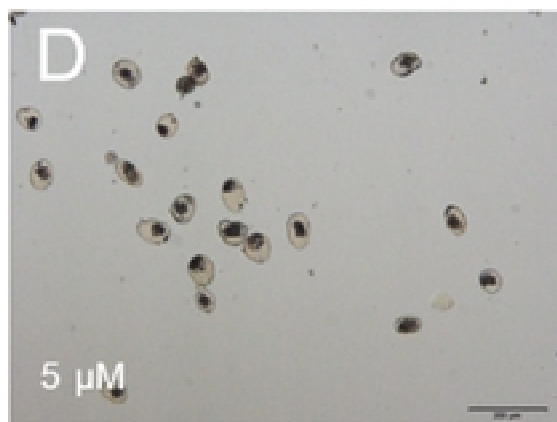
567 **Data Availability**

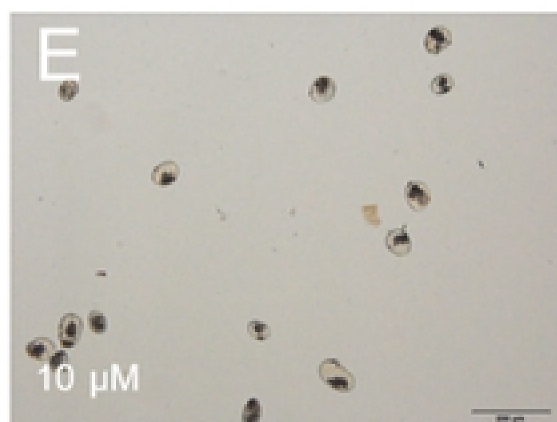
568 All relevant data are within the manuscript and its Supporting Information files.











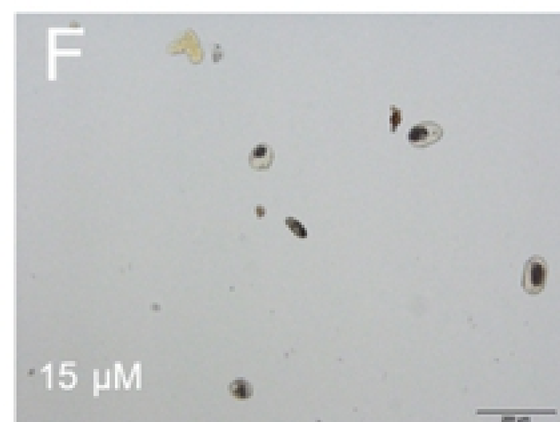


Figure.1

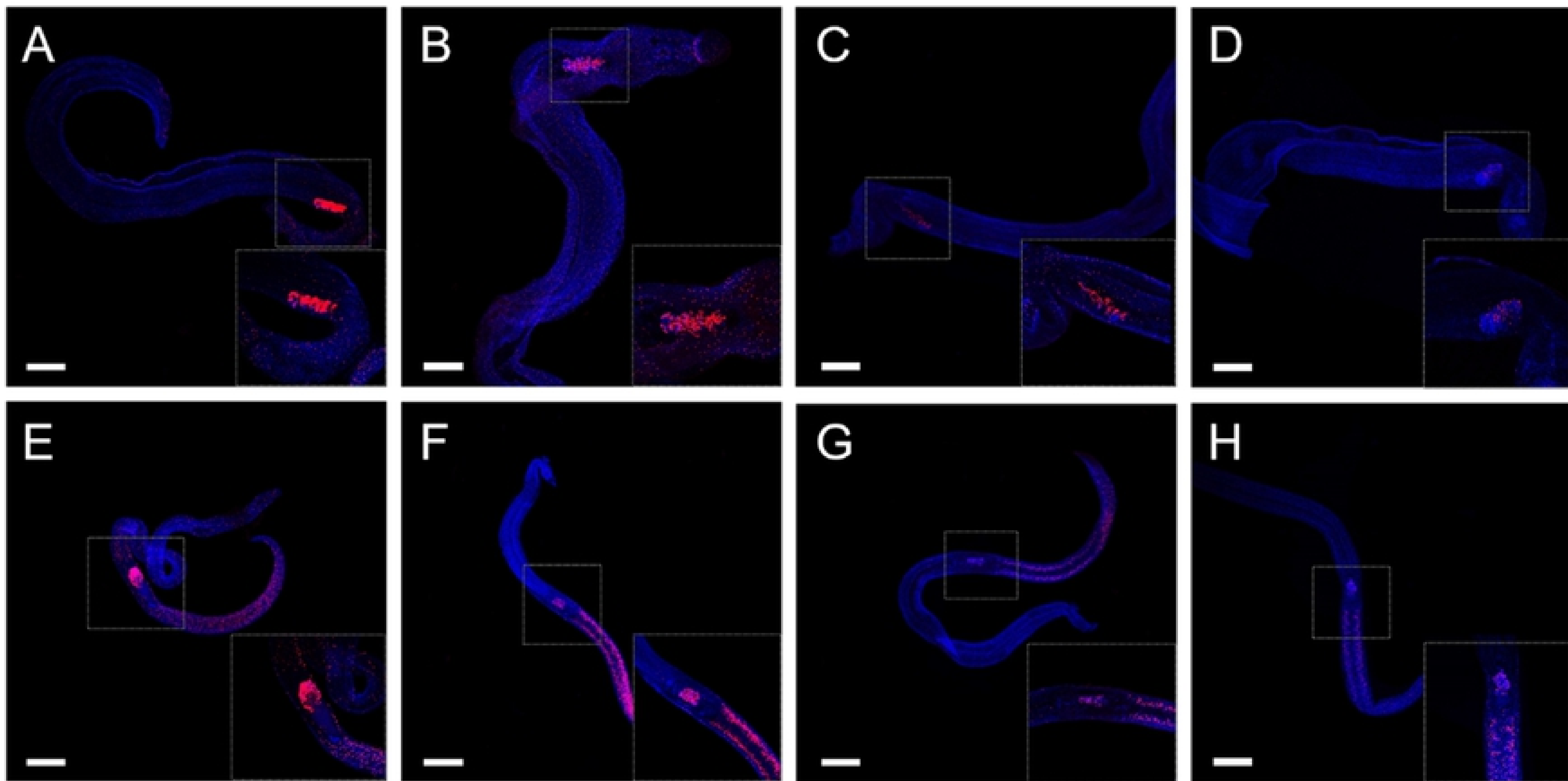


Figure.2

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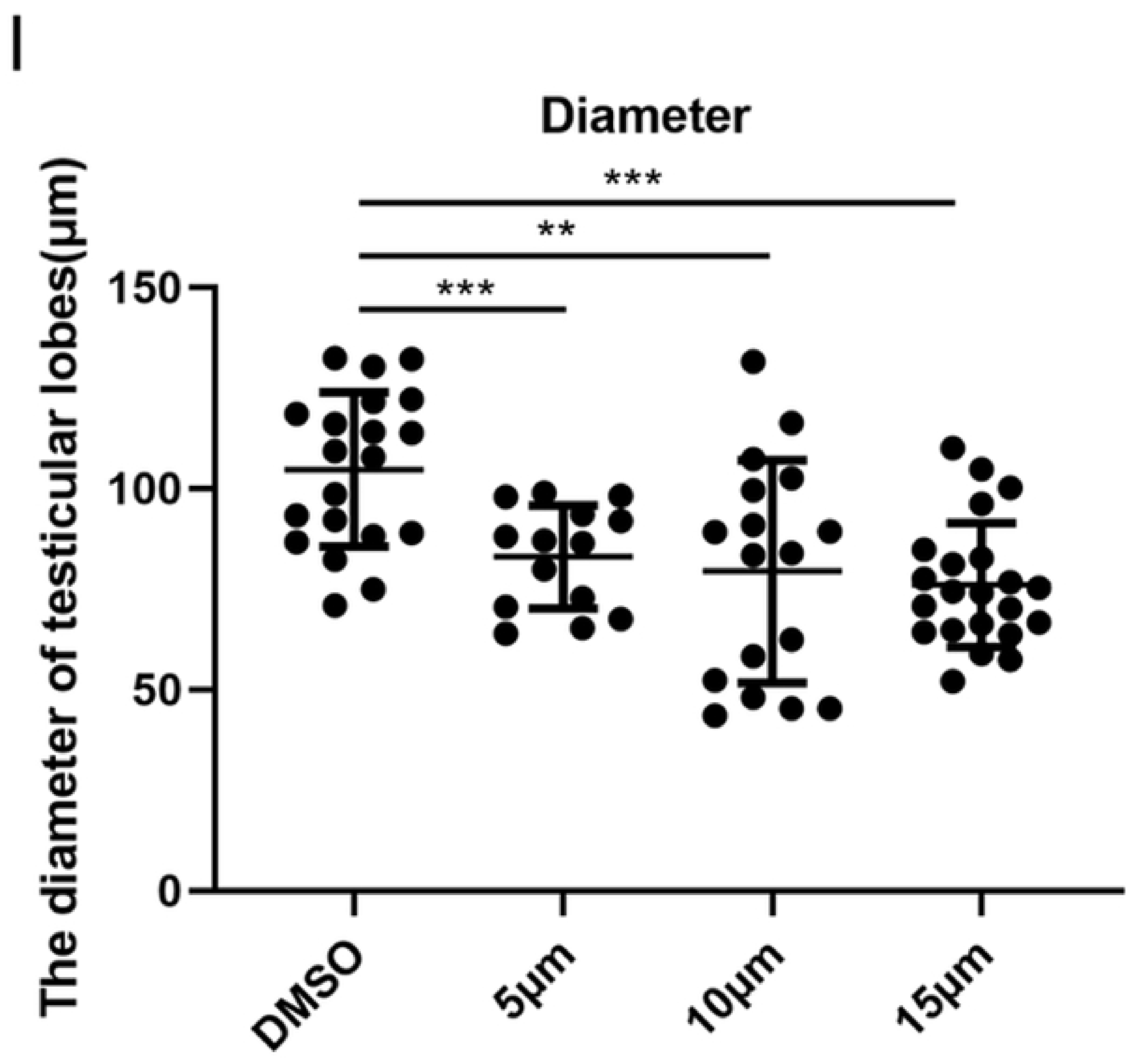


Figure.3

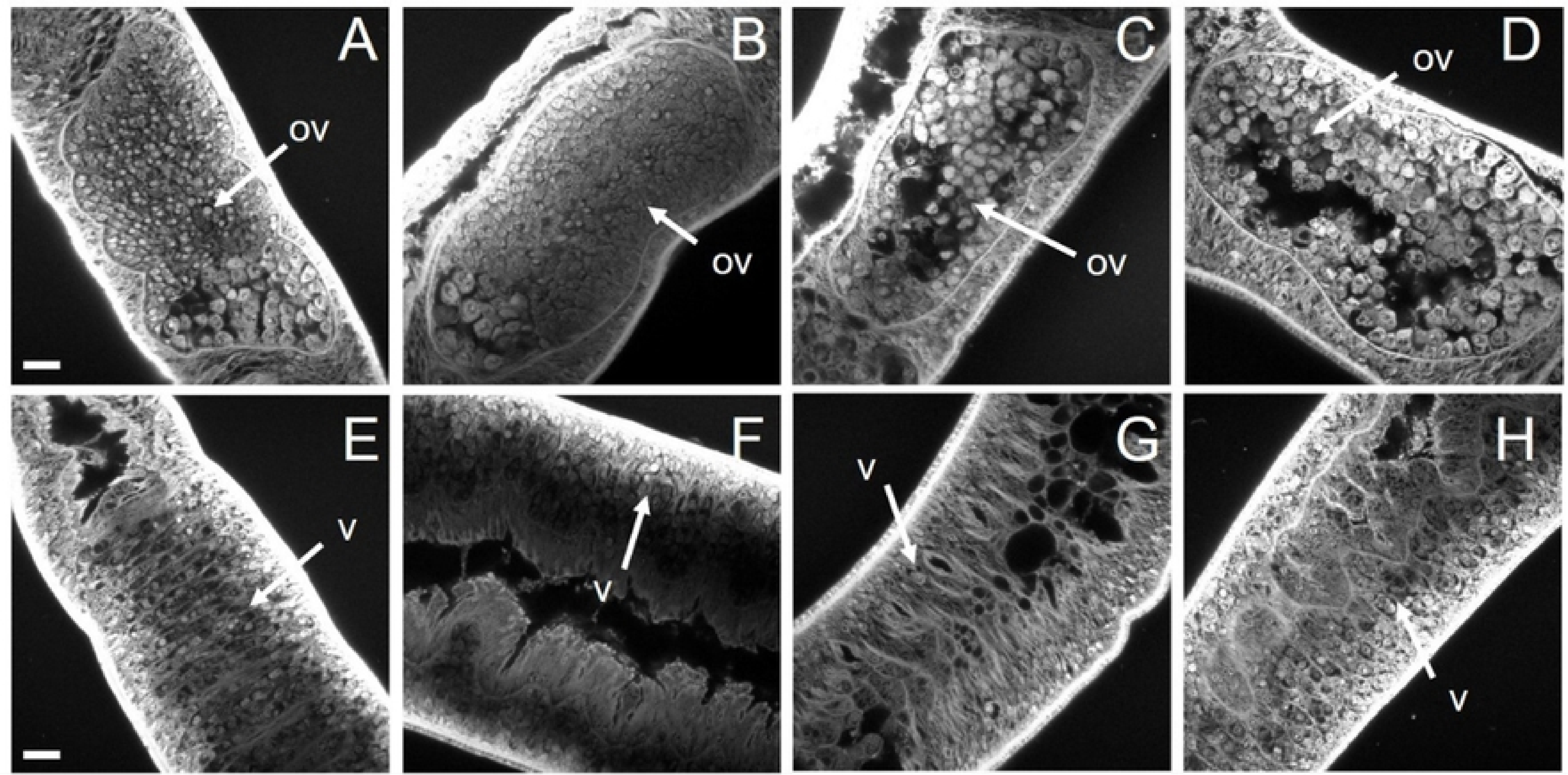
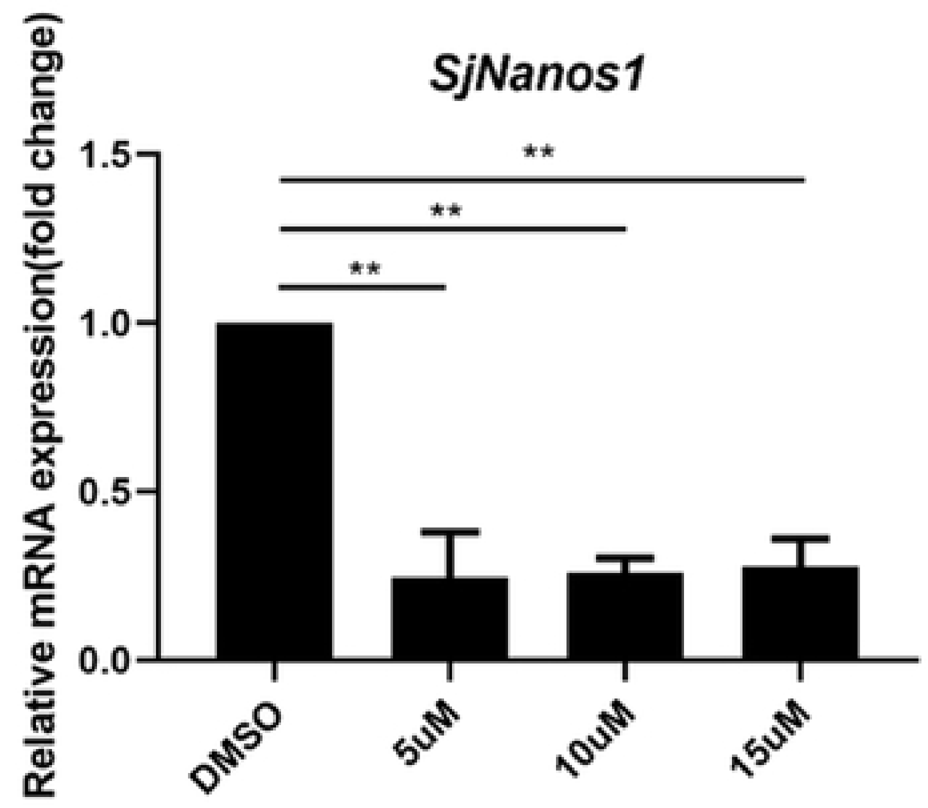
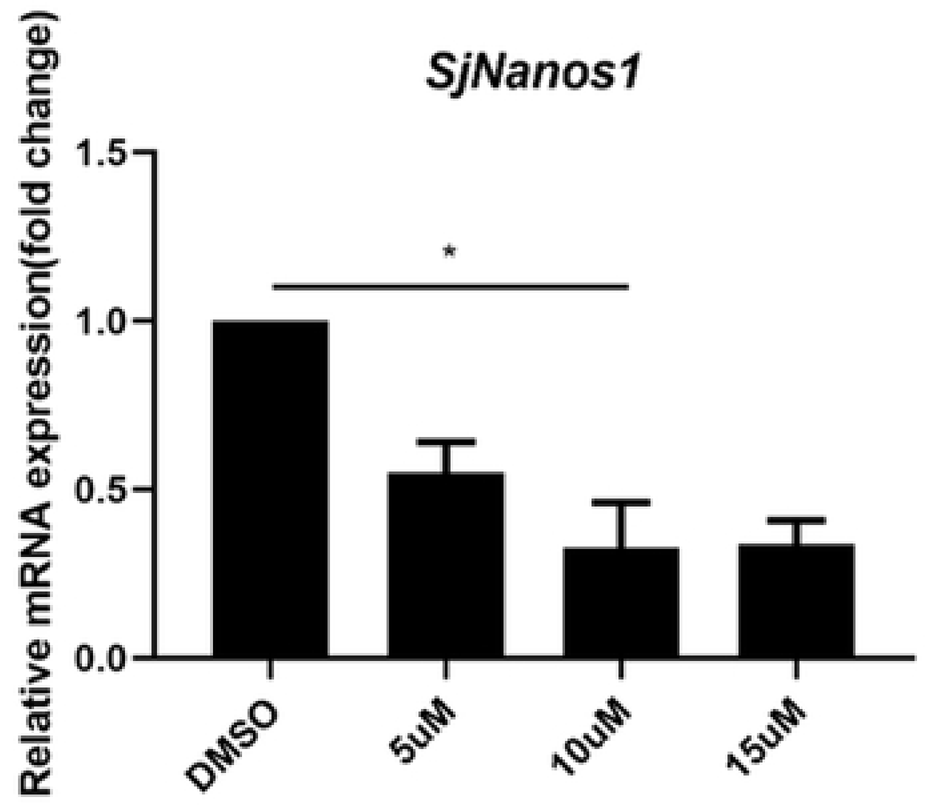
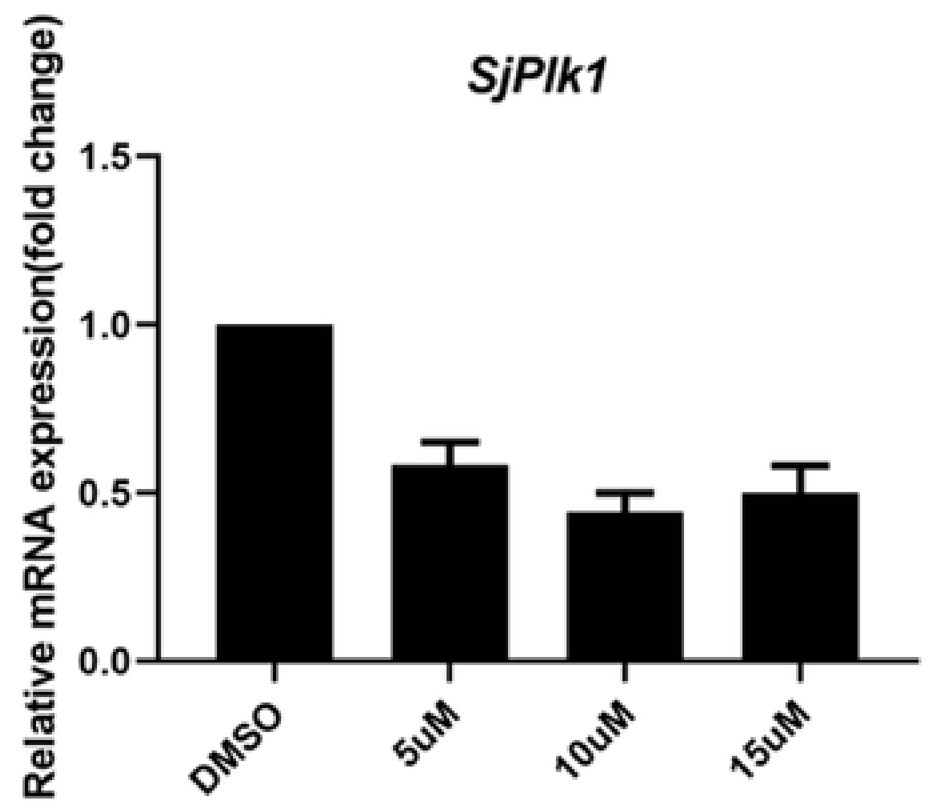


Figure.4







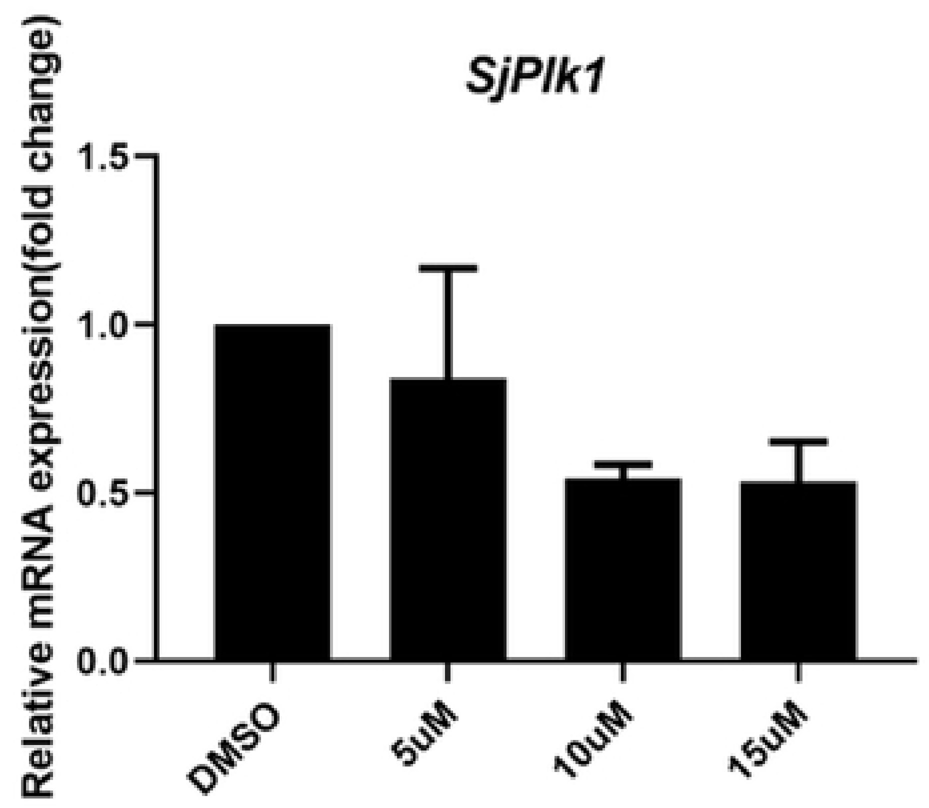


Figure.5

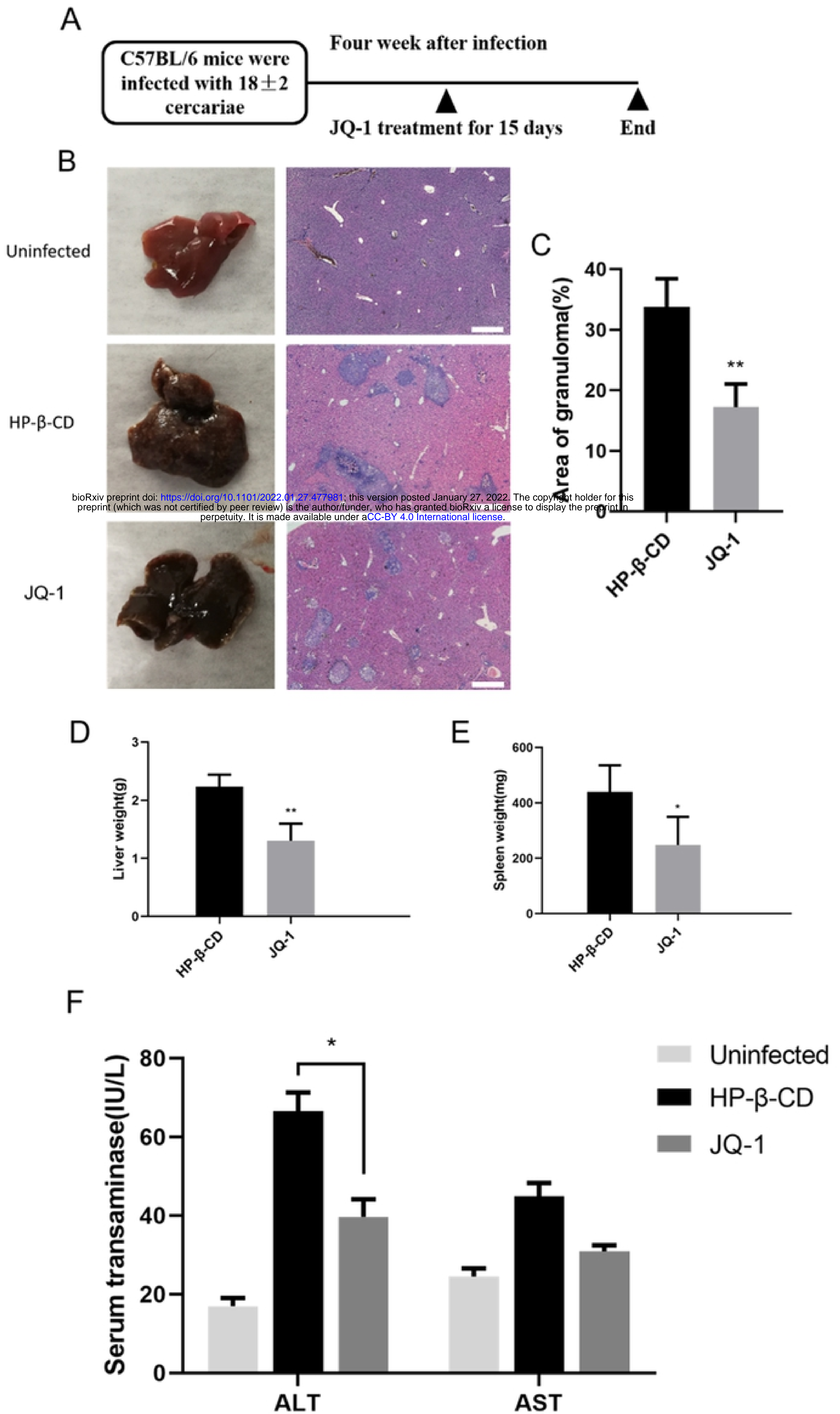


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Figure.6

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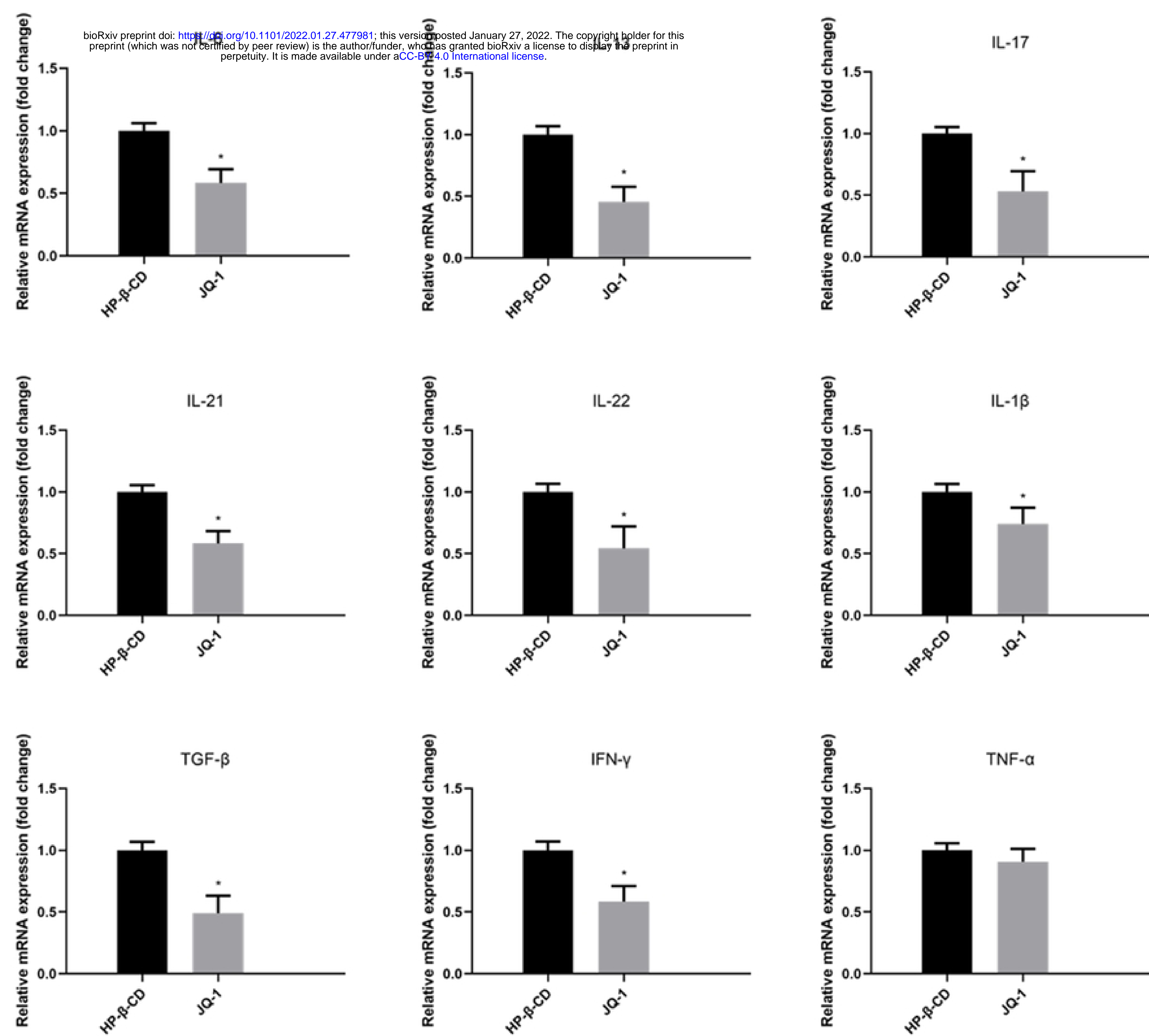

Figure.7

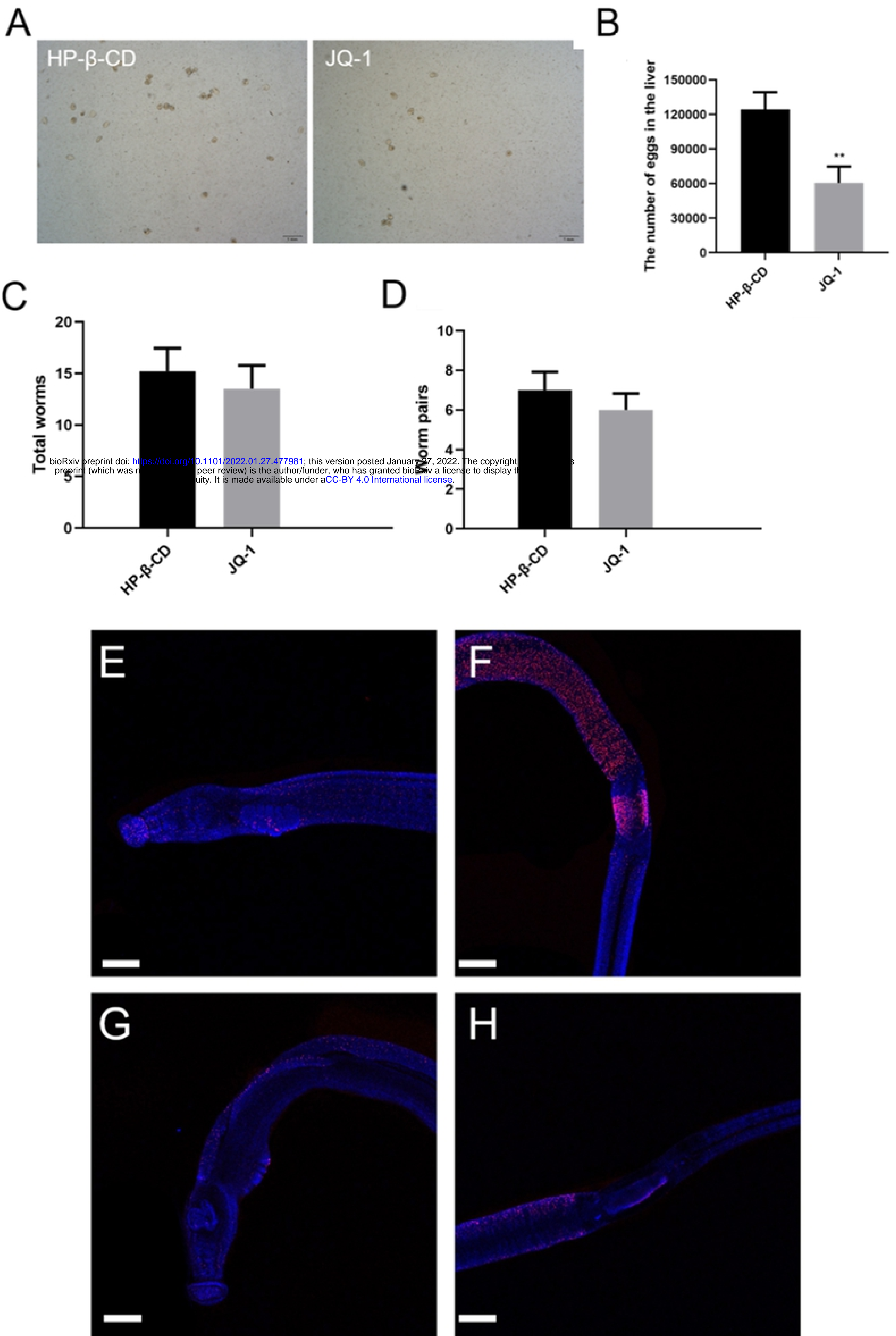

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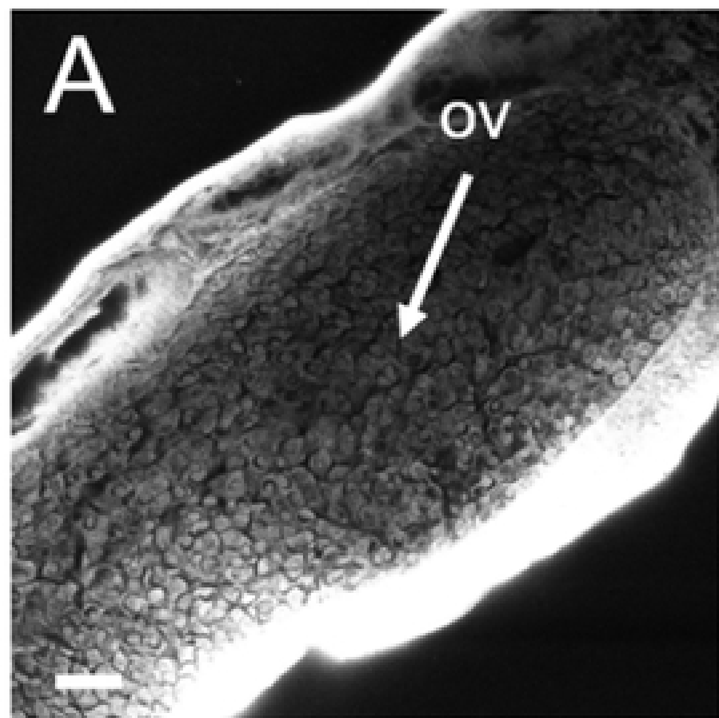
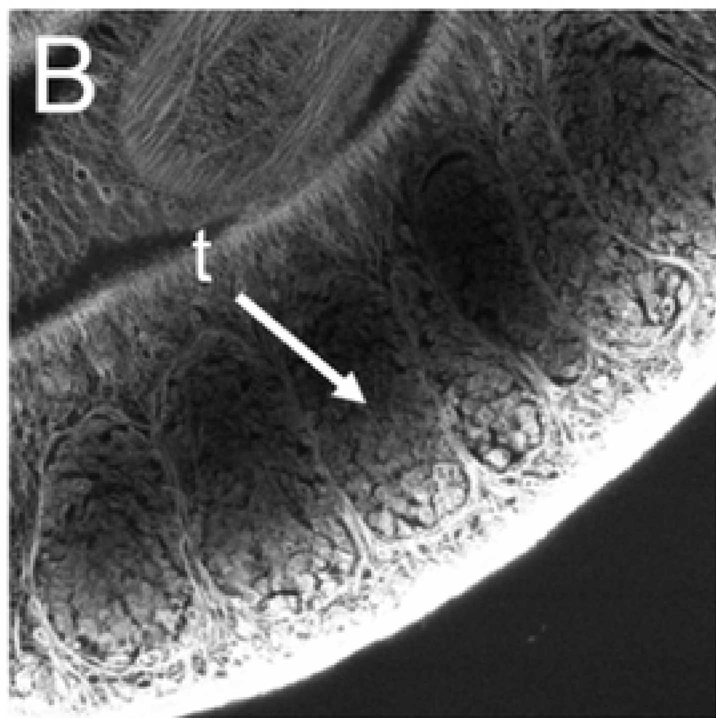
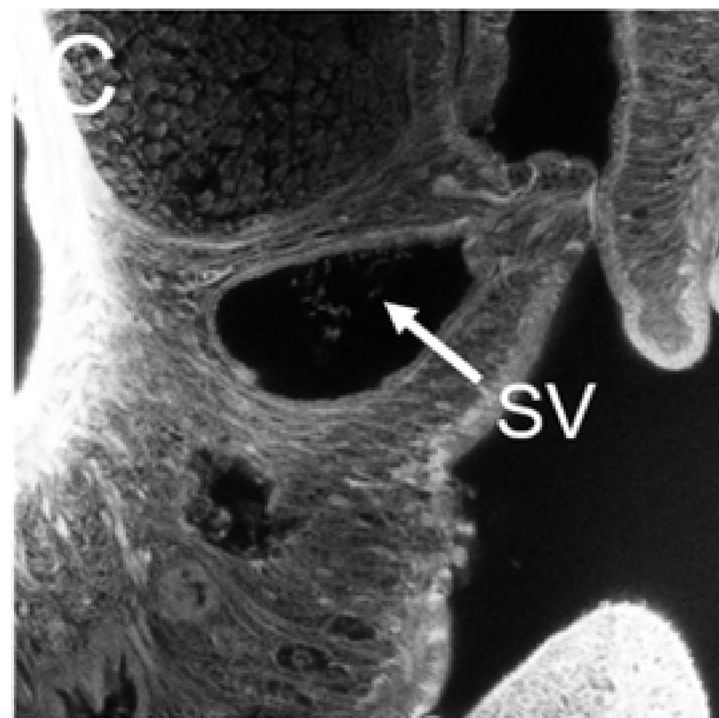
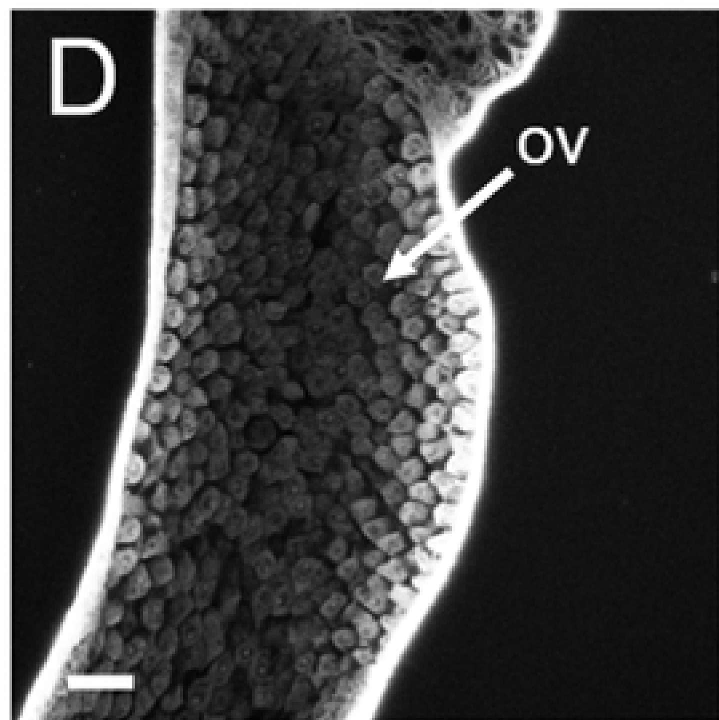
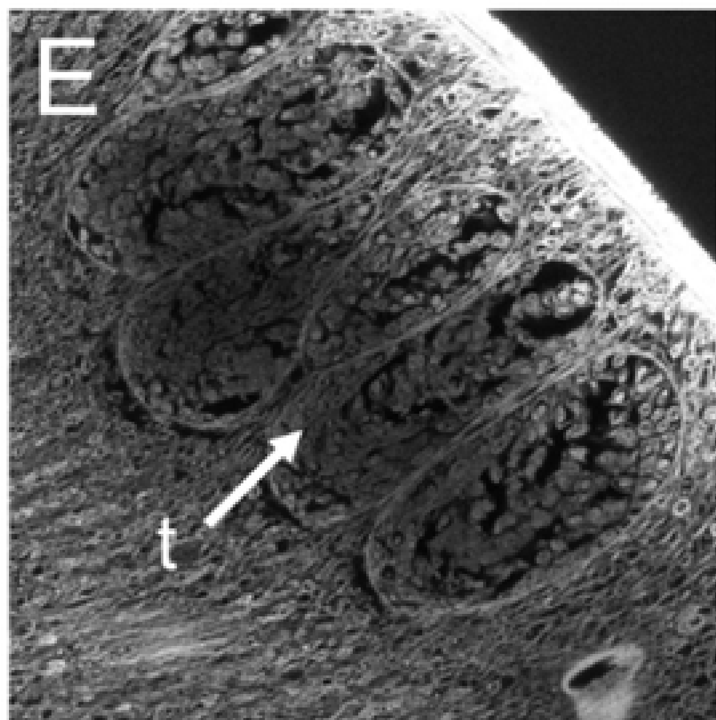
Figure.8











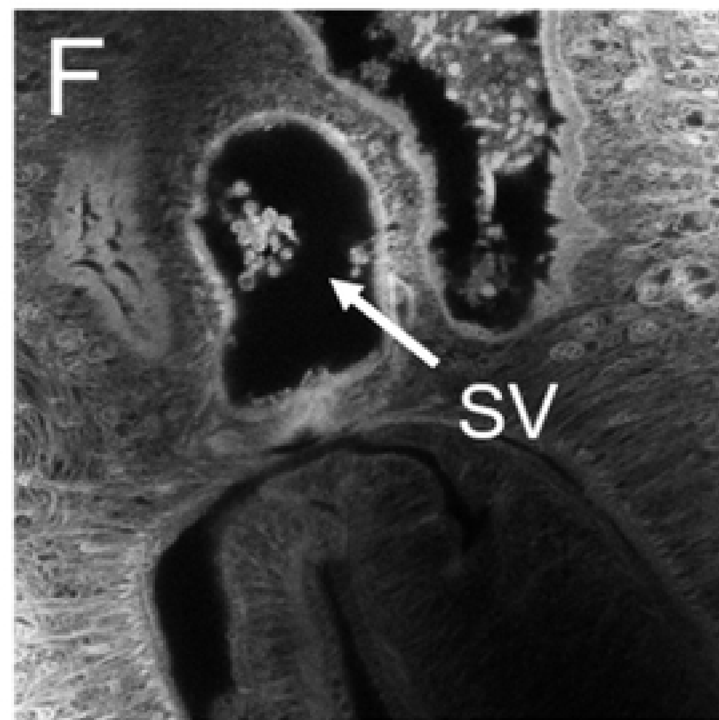


Figure.9