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Detection of histamine receptors on mouse oocytes and their involvement in fertilization potential

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Abstract

Histamine widely involves in local immune responses, physiological function in the gut, and acting as a neurotransmitter in the brain. Scientist also found the importance of histamine in the reproductive systems. The present study aimed to determine the existence of histamine receptor subtypes; *H1R*, *H2R*, *H3R*, and *H4R* on mouse oocytes through immunofluorescence (IF) staining and reverse transcription- polymerase chain reaction (RT-PCR). These further confirmed by the involvement of histamine receptor antagonists in *in vitro* fertilization (IVF). In IF staining, mouse oocytes were incubated with primary antibody against histamine receptor, followed by incubation with fluorescence conjugated secondary antibody. Then RT-PCR analysis was carried out for the undetected receptors during IF for confirmation. The RT-PCR used RNA extracted from mice COCs and cumulus free oocytes. In IVF, sperm was cultured in a group of treated histamine receptor antagonists oocytes. This investigation revealed the existance of *H1R*, *H2R*, and *H3R* on mouse oocytes in IF and RT-PCR analyses. The treatment of IVF with histamine receptor antagonists (*H1R*: pyrilamine; *H2R*: cimetidine; *H3R*: thioperamide) led to a significant reduction quantity of 2-cell embryos (4.61 ± 2.44%; 5.83 ± 4.65%; 3.83 ± 1.82%, respectively) as compared with the control group (22.50 ± 6.44%). Therefore, according to the results of this study, the presence of *H1R*, *H2R*, and *H3R* on mouse oocytes possibly will suggest the involvement of histamine in fertilization.

Introduction

It has been demonstrated that, histamine is one of the essential biogenic amines in medicine and biology. Histamine, [2- (4-imidazolyl)- ethylamine] is a low molecular weight amine synthesized from L-histidine, exclusively by L-histidine decarboxylase (HDC). Histamine is produced primarily by mast cells, basophils, histaminergic neurons in the basal ganglia of the brain, and enterochromaffin-like cells (ECL) in the stomach.[1-4] These cells are thought to be a major producers of histamine. For example, in the response of isolated peritoneal mast cells to polymyxin B, these cells were degranulated and released the stored histamine upon external stimulation.[5,6] The action of histamine is by binding to its receptor embedded on the cell membrane.[7-9] The receptors are divided into 4 groups; histamine H1 receptor (*H1R*), histamine H2 receptor (*H2R*), histamine H3 receptor (*H3R*), and histamine H4 receptor (*H4R*). Stimulation of the H1R leads to contraction of tracheal and vascular smooth muscles, the elevation of vascular permeability, and stimulation of sensory nerve endings.[10] Histamine H2 receptor, has been well-described mainly involving as a mediator of gastric acid secretion in the stomach. Meanwhile, *H3R* can act as an autoreceptor, controlling histamine synthesis and release, or a hetero-receptor, regulating the release of neurotransmitters, such as acetylcholine, dopamine, noradrenaline, serotonin, glutamate, substance P, and gamma-aminobutyric acid.[11,12] Histamine H4 receptor, *H4R* is expressed by immunologically relevant tissues, such as the spleen, thymus, mast cells, and eosinophils, and exhibit as immunomodulatory functions.[13,14]

Numerous pieces of evidence have accumulated suggesting that histamine is involved in the reproductive system. In the male reproductive system, *H1R* and H2R

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have been found on Leydig cells of mouse, rat, human, and the ectothermic vertebrate, *Hemidactylus flaviviridis*.[15-20] Pap *et al.* had reported changes in reproductive functions and sex steroid secretion in male HDC-knock out mice, probably due to the lack of peripheral histamine effect.[21] Meanwhile, Safina *et al*., demonstrated the release of histamine from sperm by an *in vitro* acrosome reaction induced by a calcium ionophore, A23187.[22] Therefore, this study was conducted for the first time to investigate the presence of histamine receptor subtypes on mouse oocytes and the involvement of these receptors in fertilization potential.

Materials and Methods

Retrieval of oocytes. Female mice, Institute of Cancer Research (ICR) strain, 8-12 weeks old were superovulated by intraperitoneal injection of 10.00 IU, Pregnant Mare's Serum Gonadotropin (PMSG; Intervet, Madison, USA), followed, 48 hr later, by administration of 10.00 IU, human chorionic gonadotropin (hCG; Intervet). At 15 to 16 hr after receiving hCG injections, the superovulated mice were sacrificed by cervical dislocation. The oviducts were excised, and the swollen ampulla was punctured to release the cumulus oocytes complexes (COC) into the prepared Hepes-buffered Toyoda Yokoyama Hoshi (hTYH) medium. The cumulus oocyte complexes (COCs) were then transferred into a 1.50 mL microcentrifuge tube and cumulus cells were removed with 10.00 µL hyaluronidase (80.00 IU; Sigma Aldrich, Burlington, USA) to obtain cumulus free oocytes. A total of 32 COCs and 200 of cumulus free oocytes were harvested for this study. Approval for this experiment was granted by the Institutional Animal Care and Use Committee (IACUC), University of Malaya, with the ethic number, FAR/3/03/2015/FSB(R).

Immunofluorescence. A total of 12 COCs were immediately washed in 1.00 M of phosphate-buffered saline (PBS; Sigma Aldrich, St. Louis, USA). The sample was fixed in 4.00% of paraformaldehyde (Sigma Aldrich) at room temperature for 2 hr and washed in 1.00 M PBS. The COCs were then adhered to poly-L-lysine coated slides (Thermo Fisher Scientific, Waltham, USA) and left for overnight in the incubator (5.00% CO_2 at 37.00 ˚C). The COCs were permeabilized with 0.20% Triton X-100 (Sigma Aldrich, Burlington, USA) in 1.00 M PBS for 10 min at room temperature. Non-specific immunoreactions were blocked with 3.00% of bovine serum albumin (Sigma-Aldrich) in 0.10 M PBS for 1 hr at room temperature and washed in 1.00 M PBS. The cells were incubated with rabbit polyclonal primary antibody directed against histamine H1 receptor (Santa Cruz Biotechnology Inc., Santa Cruz, USA); affinity-purified goat polyclonal primary antibody directed against histamine H2 receptor (Santa Cruz Biotechnology Inc.); affinity-purified goat polyclonal primary antibody directed against histamine H3 receptor (Santa Cruz Biotechnology Inc.), and rabbit polyclonal primary antibody directed against histamine H4 receptor (Santa Cruz Biotechnology Inc.). The primary antibodies were diluted in 1.00 M PBS at a 1:100 ratio. The incubation was carried out overnight at 4.00 ˚C in a humidified petri dish. After washing in 1.00 M PBS, the cells were incubated with the secondary antibody, mouse anti-goat IgG, goat anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugate (Thermo Fisher Scientific) in 1:400 dilution ratio in 1.00 M of PBS. The incubation took place for 2 hr at 4.00 ˚C in a dark, humidified petri dish before being washed in 1.00 M PBS. Negative control was prepared by incubation with the secondary antibody only. Samples were then covered with UltraCruz aqueous mounting medium (Santa Cruz Biotechnology Inc.) with 4', 6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology Inc.). Coverslip was placed carefully onto the cells before observation using a Digital Imaging Head Fluorescent Microscope (Nikon, Tokyo, Japan).

RT-PCR. Total RNA was extracted from fresh 20 COCs and 200 of cumulus free oocytes. The RNA was also obtained from the skin, stomach, brain, and testis of the mouse, which served as positive controls. The RNA extraction was performed using a TRIzol method. One milliliter of TRIzol (Thermo Fisher Scientific) was added immediately into the samples. The tube consisted of the samples and 1.00 mL TRIzol was added with 200 µL of chloroform and mixed vigorously for 15 sec. The tube was incubated for 2 to 3 min at room temperature and centrifuged at 12,000 *g* in 4.00 ˚C for 15 min. The colorless upper aqueous phase containing the RNA, 600 µL was carefully transferred to a clean tube. Next, 500 µL of cold isopropyl alcohol and 1.00 µL of glycogen (20.00 µg μL^{-1}), were added into the tube. The tube was then mixed vigorously and incubated at -20.00 ˚C overnight. The tube was centrifuged at 12,000 *g* in 4.00 ˚C for 10 min. Supernatant at the upper phase was discarded slowly and thoroughly using pipette. The RNA pellet was washed with 1.00 mL of cold 75.00% ethanol diluted in diethyl carbopyronate (DEPC) treated water. The tube was centrifuged at 9,500 *g* in 4.00 ˚C for 5 min. Ethanol was discarded carefully using a pipette. The tube was spin down shortly to collect the remaining ethanol at the bottom of the tube. The residual ethanol was discarded thoroughly and completely by using a fine tip pipette. The tube was left to air dry for 5 min at room temperature. The RNA was dissolved entirely in 30.00 - 60.00 µL of diethyl carbopyronate (DEPC; Sigma Aldrich) treated water by incubation at 60.00 ˚C for 10 min. Total RNA isolated was reverse transcribed using the QuantiNova Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions in a final volume of 20.00 µL reaction

mixture. The prepared genomic DNA (gDNA) removal reaction was incubated in thermal cycler at 45.00 ˚C for 2 min, followed by 25.00 ˚C for 3 min, 45.00 ˚C for 10 min, and 85.00 ˚C for 5 min to inactivate the reverse transcription enzyme in reverse transcription mixture. The PCR amplification was carried out in the presence of 1.00 µL of cDNA (~ 100 ng), 0.50 µM of each oligonucleotide primer (Table 1), 2.00 mM $MgCl_2$, 0.20 mM dNTPs (dATP, dTTP, dCTP, dGTP), and 0.05 units per µL of Taq DNA polymerase (Promega, Madison, USA). Expression of *Beta Actin* (*ATCB*) primers (5'- GTGGGCC GCTCTAGGCACCAA-3') and 5' CTCTTTGATGTCACGCA CGATTTC -3') which gave a 539 bp PCR product, served as an internal control for RT-PCR process.[23] The PCR master mix was prepared for each primer; *ATCB*, histamine receptor subtypes *H2R*, *H3R*, and *H4R* genes, containing all the components except the cDNA template. Later, 1.00 µL of template DNA (~ 100 ng) was added to each tube to give the final volume of 25.00 µL prior to PCR amplification. Parameters for PCR include an initial denaturation step at 94.00 ˚C for 5 min, followed by 35 cycles at 94.00 ˚C for 30 sec, 60.00 ˚C for 30 sec, and 72.00 ˚C for 1 min. The PCR products were electrophoresed on a 1.00% agarose gel stained with GelStain (Transgen Biotech, Beijing, China), and the approximate size of the amplified products was determined by referring to a 100 bp or 1.00 kb bp DNA ladder (Promega). The PCR product was viewed under a gel documentation system (Multi Image II AlphaImager HP; Alpha Innotech, San Leandro, USA). The sequencing of DNA was carried out to verify the amplified PCR products.

***In vitro* fertilization (IVF).** The COCs were divided into three groups and treated separately with 20.00 nM of pyrilamine (*H1R* antagonist; Sigma Aldrich), cimetidine (*H2R* antagonist; Sigma Aldrich), and thioperamide (*H3R* antagonist; Sigma Aldrich) in Toyoda Yokoyama Hoshi (TYH) medium covered with mineral oil. Sperm, 5.00 - 10.00 µL with a concentration of 1.00 - 2.00 × 10^6 sperm per mL, was then inseminated to each group. The culture was incubated in 5.00% CO_2 at 37.00 ˚C for 24 hr. Three IVF replicates were carried out for each treatment group. Fertilized oocytes were pooled and washed in three times of hTYH medium. The formation of 2-cell embryos was observed under inverted microscope (Olympus, Tokyo, Japan) at 40× magnification.

Statistical analysis. The analysis was carried out with SPSS Software (version 26.0; IBM Corp., Armonk, USA) version. The data are presented as the mean ± standard error of the mean. All data were analyzed using Mann Whitney U test and the number of 2-cell embryos was compared among different IVF treatment groups. A significant difference among factors was determined when the *p*-value was less than 5.00%.

Results

Detection of histamine receptor subtypes *H1R*, H2R, *H3R*, and *H4R* on mouse oocytes by immunofluorescence. The histamine H1 receptor, *H1R*, was detected on mouse oocytes, where the majority of the fluorescence signal, FITC was observed within the cytoplasm and cumulus cells of mouse oocyte (Fig. 1A). While other histamine receptors *H2R* (Fig. 1B), *H3R* (Fig. 1C), and *H4R* (Fig. 1D), were not detected on mouse oocytes by immunofluorescence.

Expression of histamine receptor mRNAs on mouse oocytes. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was carried out for the undetected receptors during IF for confirmation presence of histamine receptors on mouse oocytes. Agarose gel electrophoresis (1.00%) showed the amplification of expected PCR products *H2R* (442 bp) on mouse COCs (Fig. 2A) while Figure 2B did not show the expected *H2R* PCR products on mouse cumulus free oocytes. The results also showed the amplification of expected PCR products *H3R* (282 bp) on mouse COCs (Fig. 2C) and *H3R* on cumulus free oocytes (Fig. 2D). No amplification of the PCR product for *H4R* on both COCs (Fig. 2E) and cumulus free oocytes (Fig. 2F).

Effects of histamine receptor subtypes antagonists in *in vitro* fertilization. Two-cell embryos was observed after 24 hr of incubation (Fig. 3). The number of 2-cell embryos was significantly reduced ($p < 0.05$) in *H1R* antagonist (4.61 ± 2.44%), *H2R* antagonist (5.83 ± 4.65%), and *H3R* antagonist (3.83 ± 1.82%) treated groups in comparison with the control group (22.50 ± 6.44%). The bar graph showed the total of 2-cell embryos in *H1R* antagonist, *H2R* antagonist, and *H3R* antagonist treated groups were lower than the control group (Table 2).

Table 1. Primer sequences and expected size of amplicons.

Genes (Accession No.)	Primer sequence (5'-3')	Amplicon size
***H1R* (NM_001010973.2)**	Forward: GCAGCACCAGCTCCTATGAC Reverse: GCCCTGTGGCTTCTACACTC	442 bp
***H2R* (NM_001010973.2)**	Forward: AGGATGGAACAGCAGAAAT Reverse: TGTCCTGACTGGGCTTCCCT	610 bp
***H3R* (NM_133849.3)**	Forward: CCATTGCACAGGTATGGGGT Reverse: CACGATGATAGCCAGCGACT	282 bp
***H4R* (NM_153087.2)**	Forward:CCTGTCATCTCTGTGGCTTATT Reverse:AGCTCTCAGTCTGTCATGTTTC	806 bp

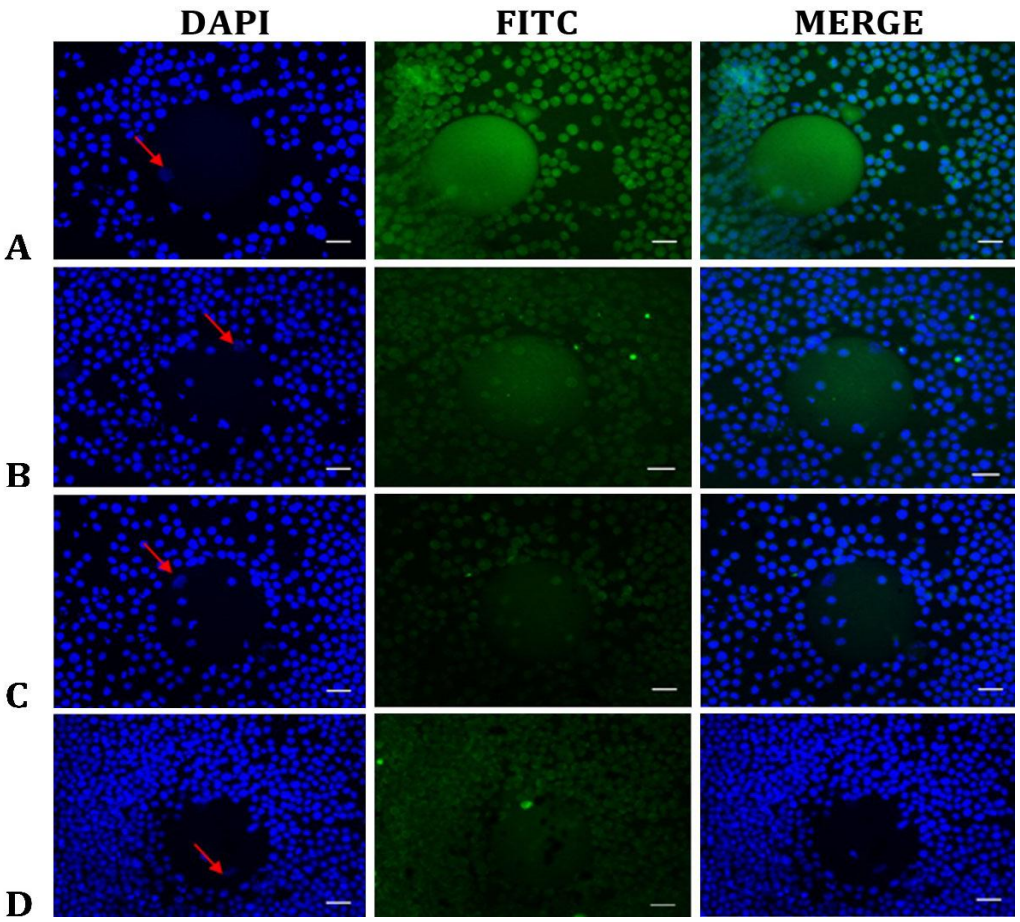

Fig. 1. Immunofluorescence detection of the histamine receptor subtype on mouse cumulus oocyte complex (COC). **A)** COC incubated with the antibody against *H1R*; **B)** COC incubated with the antibody against *H2R*; **C)** COC incubated with the antibody against H3R; **D)** COC incubated with the antibody against *H4R*. Red arrows indicate the nucleus within the cytoplasm of the oocyte (bars: 10.00 µm).

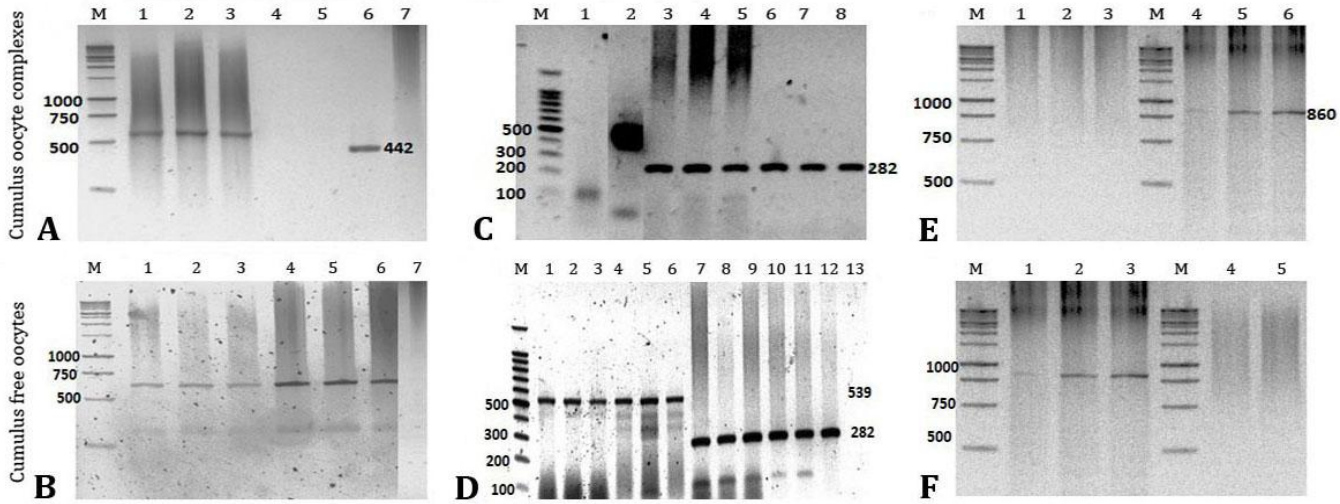

Fig. 2. Agarose gel electrophoresis shows the expression of histamine receptors *H2R* (**A**, **B**), *H3R* (**C**, **D**), and *H4R* (**E**, **F**) genes on mouse cumulus oocyte complex (COCs) and cumulus free oocytes (CFO). M: 100 bp DNA ladder. **A)** Lanes 1-3: *ACTB* on COC (539 bp); Lanes 4-6: *H2R* on COC (442 bp); Lane 7: Negative control. **B)** Lanes 1-3: *H2R* on stomach (610 bp); Lanes 4-6: H2R on CFO (610 bp); Lane 7: Negative control. **C)** Lane 1: Negative control; Lane 2: *ACTB* on COC (539 bp); Lanes 3-5: *H3R* on brain (282 bp); Lanes 6-8: *H3R* on COC (282 bp). **D)** Lanes 1-3: *ACTB* on brain (539 bp); Lanes 4-6: *ACTB* on CFO (539 bp); Lanes 7-9: *H3R* on brain (282 bp); Lanes 10-12: *H3R* on CFO (282 bp); Lane 13: Negative control. **E)** Lanes 1 and 2: *H4R* on COC; Lane 3: Negative control; Lanes 4-6: *H4R* on testis (806 bp). **F)** Lanes 1-3: *H4R* on testis (806 bp); Lane 4: *H4R* on CFO; Lane 5: Negative control.

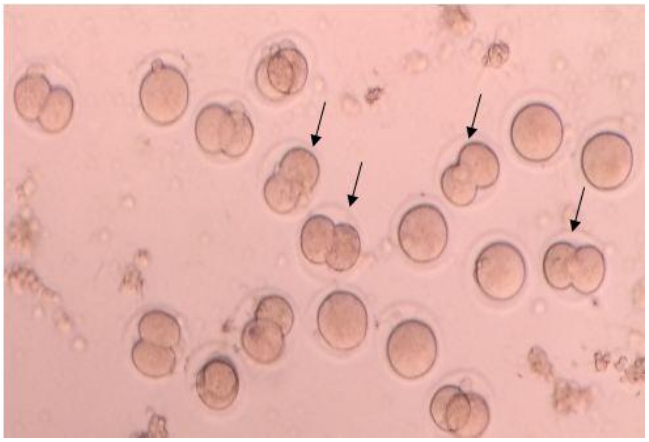

Fig. 3. Formation of 2-cell embryos (black arrows), (40×).

Table 2. Statistical analysis number of 2-cell embryos in control and histamine receptor antagonist treated groups.

Groups	Total oocytes	No. (%) of 2-cell embryo	Mean ± standard error of the mean
Control	210	70 (33.00)	22.50 ± 6.44[a]
***H1R* antagonist**	216	12 (5.50)	4.61 ± 2.44[bc]
***H2R* antagonist**	195	19 (9.70)	5.83 ± 4.65[b]
***H3R* antagonist**	217	6 (2.80)	3.83 ± 1.82[c]

[abc] Different superscripts within the same column show a significant difference at $p < 0.05$.

Discussion

The study of histamine in fertilization has started when histamine was first found to be released from sperm by an *in vitro* acrosome reaction induced using a calcium ionophore, A23187. Later, a study on the effects of histamine on invertebrates fertilization was conducted by Leguia and Wessel.[24] The authors found the presence of *H1R*, a semi-transmembrane G protein-coupled receptor on the surface of the sea urchin egg. Activation of this *H1R* had induced nitric oxide (NO) production that was essential for calcium dynamics during fertilization.

Calcium ion, Ca^{2+}, is the universal signal for egg activation during fertilization in all sexually reproducing species.[25] An increase in the intracellular concentration of Ca^{2+} underlies the initiation, progression, and completion of a wide variety of cellular processes, including fertilization, muscle contraction, secretion, cell division, and apoptosis.[26] Activation of histamine via *H1R* was found to increase the intracellular Ca^{2+} concentrations.[27] There was a possibility that inhibition of *H1R* in the present study affected the production of Ca^{2+}, which suppressed the fertilization process. The result was quite similar to the findings of Wozniak *et al.* that Ca^{2+} blocker inhibited embryonic development in a concentration-response manner in African clawed frog.[28] However, further investigation is needed, especially in mammals, necessary to explore the mechanism of histamine-induced calcium during fertilization.

Available pieces of evidence have shown the potential effects of histamine receptor antagonists or antihistamine in male reproductive functions. For example, a reduction in sperm count, as well as a decrease in sperm morphology and motility after 15-days treatment of cimetidine, a class of drug used to treat the acid-related gastrointestinal diseases, had demonstrated in Albino rats.[29] Later, the harmful effect of cimetidine on the number of sperm cells in Wistar rats was confirmed by Aprioku *et al.*[30] Cimetidine had also caused adverse effects on semen quality (count, motility, vitality, and morphology) mainly when it was used at high concentrations in men (i.e., ≥ 100 mg per day).[31] A recent study reported that cetirizine hydrochloride, a second-generation antihistamine, had significantly affected prolactin, luteinizing hormone, and follicle-stimulating hormone levels.[32] Ranitidine, a type of *H2R* antagonist, had negatively affected the frequency of sperm head abnormality and male sex hormones level [33] Other observations on the effects of antihistamine in male reproductive system are also indicative its potential to affect fertilization. In line with previous studies, the present study found the effect of antihistamine in fertilization, where the number of 2-cell embryos in histamine receptor antagonists-treated IVF was significantly reduced in comparison to the control group.

With specific concern over the male gonad, a large body of work have described the presence of histamine receptors in the diverse compartments of the testis in several species, including humans. For instance, histamine receptor, *H1R*, and *H2R* has been detected on germ and peritubular cells, as well as in macrophages, and Leydig cells where as the *H1R* was also found in the testicular capsule.[34] A previous study also found expression of *H4R* on the Leydig cells of rat, which indicated the presence of histamine H4 receptors on the Leydig cells of male testis.[35] There have been no published repost so far, to the best of the author's knowledge on the discovery of histamine receptor on mouse oocytes. The present study is to first to detect *H1R* on mouse oocytes using immunofluorescence staining. Furthermore, with more sensitive techniques, i.e., RT-PCR, the expression of histamine receptors, *H2R* and *H3R* were also found in the mouse COCs and cumulus free oocytes. The finding of *H2R* and *H3R* on mouse oocytes in the study was in agreement with Virant-Klun *et al.* on the importance of gene expression profiling on oocyte for a successful fertilization.[36]

The success of fertilization can be assessed by observing: (1) spermatozoa inside the zona pellucida (ZP); (2) the formation of pronuclei; or (3) the formation of 2-cell embryos. According to results of this study, histamine could be one of the factors associated with fertilization. This finding was in agreement with Okabe that the fertilization-related factors had been identified when antibodies or inhibitors added, successfully inhibited the fertilization *in vitro*.[37]

Therefore, according to the findings in this study, it is suggested that, histamine possibly involves in the fertilization process, where it could be conceivably one of fertilization-related factors. The histamine gene-disruption experiments in mice are recommended to be conducted in the future to confirm the tasks of histamine in fertilization. The relevant results of this current experiment can be used as a starting point to investigate the role of histamine in the fertilization process.

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Conflict of interest

The authors declare that there are no conflicts of interest related to this article.

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