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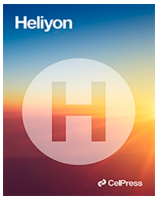

Research article

Associations between neonicotinoid insecticide levels in follicular fluid and serum and reproductive outcomes among women undergoing assisted reproductive technology: An observational study

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ABSTRACT

Neonicotinoid insecticides (NEOs) are a widely used type of insecticide found globally, leading to broad human exposure. However, there is limited research on how internal exposure levels of NEOs and their metabolites impact in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) outcomes. A study was conducted at the Sixth Affiliated Hospital of Sun Yat-sen University between 2017 and 2020 involving 436 women undergoing IVF/ICSI treatment. Data on demographics and clinical history were collected from medical records. The concentrations of 11 NEOs and 4 NEO metabolites in follicular fluid and serum were measured using a salting-out assisted liquid–liquid extraction method and liquid chromatography–tandem mass spectrometry. Our findings indicated that NEOs were prevalent in women with infertility. One NEO metabolite, N-dm-ACE, was detected in all samples with median concentrations of 0.221 ng/mL in follicular fluid and 0.228 ng/mL in serum. The study showed a decrease in the number of retrieved oocytes, mature oocytes, 2 PN zygotes, and high-quality embryos as the number of exposed NEOs in follicular fluid increased. Women in the highest tertile of N-dm-ACE exposure had fewer mature oocytes, 2 PN zygotes, and lower oocyte maturity rates compared to those in the lowest tertile. The findings suggest that exposure to NEOs may negatively impact reproductive outcomes in IVF/ICSI pregnancies, particularly affecting oocyte retrieval and embryo quality. This study highlights the potential adverse effects of environmental NEO exposure on IVF/ICSI outcomes, emphasizing the importance of considering such exposures in preconception care.

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1. Introduction

Infertility is commonly defined as the inability to achieve a successful pregnancy after 12 months of unprotected heterosexual intercourse [1]. Studies suggest that approximately one in six couples worldwide experience challenges with involuntary childlessness [2]. Alongside biological factors, lifestyle choices such as unhealthy habits and psychological stress have been linked to infertility [3,4]. Emerging research indicates that exposure to environmental endocrine-disrupting chemicals can impact human reproductive health by disrupting hormonal balance, a crucial factor in the development of infertility [5,6].

Neonicotinoid insecticides (NEOs) are a group of chemicals utilized as insecticides due to their impact on nicotinic acetylcholine receptors (nAChRs). They have gained prominence in the global market owing to their wide-ranging pest control capabilities, minimal environmental residue, and low human toxicity [7,8]. Since the 1990s, starting with imidacloprid (IMI), various other NEOs like acetamiprid (ACE), clothianidin (CLO), thiamethoxam (THX), thiacloprid (THI), nitenpyram (NIT), and dinotefuran (DIN) have been progressively developed for commercial purposes [9]. These NEOs, known for their effective pest management and safety profiles, have been widely used globally in agriculture, animal husbandry, and residential settings [10]. NEOs have the propensity to contaminate the external environment easily. Unlike contact pesticides that remain on the surface of plants, NEOs can be absorbed by plant roots and distributed to nearly all plant tissues. This leads to the presence of NEO residues in various fruits and vegetables such as carrots, leafy greens, baby cabbage, and apples, with conventional cleaning methods proving ineffective in their removal. These chemicals can disperse into the air through pollen, seep into soil and water, or persist in crops [11]. NEOs exhibit high water solubility, ranging from 185 mg/L to 600,000 mg/L at 20 °C. Consequently, NEOs are often detected in soil-water-sediment systems. While NEOs metabolize rapidly in soil, with half-life values of 1–4 days, their solubility and application in soil and seed treatments enable most NEOs to infiltrate oxygen-deficient groundwater, where they degrade slowly and accumulate [12,13]. Since 2010, NEOs have commanded a substantial share of the global insecticide market, accounting for 25 % with annual sales amounting to 1.9 billion dollars [14]. China, contributing around 70 % of IMI production, manufactures approximately 20,000 tons of the active substance annually [15]. Numerous studies have highlighted the ease with which NEOs enter ecosystems through runoff and drainage systems in agricultural regions, posing escalating ecological risks to various organisms [12,16]. The general population may be exposed to NEOs through various routes, including consumption of contaminated produce, tea beverages, and drinking water, as well as through dermal contact or inhalation during pesticide application, sowing of treated seeds, and other occupational practices. Indoor air pollution stemming from structural wood treatments for termite control and pest management for pets can also contribute to NEO exposure [17–19]. NEOs exhibit a strong affinity for binding with proteins like globular albumin and hemoglobin, which travel throughout the body via the bloodstream [20]. Their distribution spans across various organs and tissues, including the spleen, heart, bones, lungs, adrenal glands, ovaries, uterus, peripheral nerves, pancreas, thyroid, brain, liver, kidneys, skeletal muscles, adipose tissue, testes, and skin [21,22]. Upon exposure, NEOs undergo metabolization into specific metabolites through phase I enzymes and biotransformation processes such as demethylation, hydroxylation, and nitro reduction [23,24]. The majority of NEOs and their metabolites are excreted in urine, to some extent in feces, but minimally in the lungs [25].

Recent studies conducted through in vitro, *in vivo*, and ecological field investigations have highlighted the potential adverse effects of NEOs on mammals, even at sublethal concentrations [26–28]. NEOs impact mammalian nAChRs in a manner akin to nicotine [29], which play a crucial role in human brain functions encompassing development, memory, cognition, and behavior [30]. Recent *in vivo* studies have shown that NEOs like IMI and ACE can negatively affect mammalian reproductive organs by impeding testicular development, impairing spermatogenesis, reducing sperm quality, and altering ovary morphology [31–34]. Kapoor et al.[34] observed elevated serum follicle-stimulating hormone (FSH) levels and lowered levels of serum luteinizing hormone (LH) and progesterone in 1-week-old rats exposed to a 20 mg/kg dose of IMI. Their study suggested that IMI induces the generation of reactive oxygen species (ROS) by disrupting hormonal balance [34]. Nevertheless, research concerning the impact of NEO exposure on female fertility remains limited at present.

Previous epidemiological studies have predominantly utilized blood and urine as biomatrices for assessing exposure to toxic elements [35–38], with only a limited number incorporating ovarian follicular fluid as a biomarker of exposure [39,40]. Ovarian follicular fluid, which is a filtrate of blood plasma selectively excluding high molecular weight proteins, facilitated by the blood-follicle barrier, surrounds a developing oocyte within a growing follicle [41]. The composition of follicular fluid may mirror environmental exposures relevant to early stages of human reproduction, impacting the quality of developing oocytes and embryos [42]. It is believed that follicular fluid offers a more accurate representation of the biologically effective dose of a toxicant for an oocyte compared to measurements obtained from blood or urine [43]. Hence, our research aims to (1) examine the concentrations and profiles of 15 NEOs and their metabolites in follicular fluid and serum of women with infertility in South China, and (2) evaluate their effects on assisted reproductive outcomes. To the best of our knowledge, this study represents the first investigation of NEO exposure in women with infertility in South China.

2. Materials and methods

2.1. Study population

As part of the basic research project focusing on establishing and utilizing assisted reproductive population and offspring cohorts in the Chinese population (2017YFC1001000) [44], our study involved the recruitment of women with infertility who underwent an IVF/ICSI cycle at the Sixth Affiliated Hospital of Sun Yat-sen University between January 2017 and January 2020 and resided in

Guangzhou during their IVF/ICSI treatment period. Inclusion criteria comprised meeting infertility diagnostic criteria with indications for IVF/ICSI, while exclusion criteria encompassed congenital gonadal hypoplasia, chromosomal abnormalities, familial hereditary diseases, ongoing treatment with antitumor drugs, radiation, or chemotherapy, exposure to other harmful toxins pre-assisted reproductive therapy, history of oophorectomy, genital malformations, renal or liver dysfunction, and acute or chronic infections. Prospective collection of biological samples was conducted on each participating couple following comprehensive consultation, physical examinations, and laboratory evaluations. Subsequently, women were monitored throughout their IVF/ICSI cycles until achieving a live birth or discontinuation of treatment at the Center for Reproductive Medicine. A total of 436 women were included in the study, which received approval from the Ethics Committee of The Sixth Hospital of Sun Yat-sen University (2024ZSLYFEC-006) and strictly adhered to the principles of the Declaration of Helsinki. Prior to sample collection, all women provided informed consent.

2.2. IVF/ICSI treatment procedure

The IVF/ICSI procedure involves four main steps: controlled ovarian hyperstimulation (COH), oocyte retrieval, embryo transfer, and pregnancy testing. During COH, multiple ovarian follicles are stimulated to promote ovulation. Female participants underwent specific COH protocols tailored to their ovarian response, which could include long GnRH agonist, antagonist, or short agonist protocols. Follicle development was regularly monitored using transvaginal ultrasound, and levels of serum estradiol (E2), progesterone, and LH were evaluated throughout the cycle. Administering a dose of 0.25 mg of recombinant human chorionic gonadotropin (rHCG) triggered final maturation once at least one leading follicle reached 18 mm or when three follicles reached 17 mm. Oocyte retrieval was then performed transvaginally under ultrasound guidance 36 h post-rHCG injection, followed by either IVF or ICSI fertilization based on the specific requirements of each case.

Embryologists, who were blinded to the study details, assessed the numbers of retrieved mature oocytes (metaphase II, MII), oocytes with two pronuclei (2 PN), and good-quality embryos. Embryo culture adhered to standard protocols and was evaluated using the international morphological grading system, specifically the Peter scoring system. Cleavage embryos graded as 1 or 2 with a minimum of 5 blastomeres were classified as transferable embryos, while those graded as 1 or 2 with 6–10 blastomeres were deemed good-quality embryos [45]. Blastocysts were evaluated utilizing the Gardner scoring system, with embryos graded as 3BB and above considered good-quality blastocysts [46].

Fresh embryo transfers (FETs) were conducted 3–5 days post-oocyte retrieval, with the number of embryos transferred determined by factors such as the woman's age, embryo quality, and overall health considerations. If there was an early increase in progesterone levels, the risk of moderate to severe ovarian hyperstimulation syndrome, or inadequate endometrial development, the FET procedure would be halted, and all embryos would be cryopreserved. Subsequent frozen-thawed embryo transfers (TETs) could occur in a natural cycle with or without human chorionic gonadotropin (HCG) or in an artificial cycle utilizing oral estradiol. Luteal support would be administered until a negative HCG test on day 14 or confirmation of intrauterine pregnancy via ultrasound on day 35 post-embryo transfer.

2.3. Outcome assessment

Key intermediate outcomes of the study encompassed the number of retrieved oocytes, MII oocytes, 2 PN zygotes, good-quality embryos, oocyte maturity rate, fertilization rate, cleavage rate, and good-quality embryo rate. The oocyte maturity rate was determined by dividing the number of mature oocytes by the total retrieved oocytes. Fertilization rate was calculated by dividing the number of 2 PN zygotes by the total number of IVF or ICSI oocytes. Cleavage rate was obtained by dividing the number of cleavage-stage embryos by the number of fertilized oocytes. The percentage of good-quality embryos was calculated by dividing the number of good-quality embryos by the total number of 2 PN zygotes.

Pregnancy outcomes were evaluated in the cohort of women who underwent their initial embryo transfer ($n = 359$). The clinical endpoints examined encompassed biochemical pregnancy, clinical pregnancy, miscarriage, and live birth rates. Biochemical pregnancies were confirmed by a serum β-hCG level exceeding 5 mIU/mL at 12 days post-blastocyst transplantation or 14 days post-cleavage-stage embryo transplantation. Clinical pregnancy was ascertained by the presence of a gestational sac with a discernible fetal heartbeat observed on ultrasound three weeks following embryo transfer. Miscarriage was defined as the spontaneous termination of a pregnancy before 28 weeks gestation with a fetal weight below 1000 g. Live birth denoted the delivery of a viable infant at or beyond 28 weeks gestation.

2.4. Sample collection

For women with infertility, blood and follicular fluid samples were obtained for analysis. Follicular fluid was extracted from 2 to 4 follicles per woman during oocyte retrieval and pooled in a 15 mL Falcon tube. The fluid underwent visual inspection to exclude samples with blood contamination, with only clear fluid being subjected to analysis. Following centrifugation at 2000 rpm for 10 min, the supernatant was preserved in polypropylene tubes at −80 °C until required for analysis. Blood samples, collected in the morning of HCG administration, were processed by centrifuging whole blood at 3000 rpm for 10 min at 4 °C. The resulting serum was divided into 2.0 mL aliquots, treated with methanol solution, and placed in labeled cryotubes for air drying. Subsequently, the labeled samples were frozen at −80 °C until further analysis.

Table 1
Demographic and clinical characteristics of all the participants.

Characteristic	Mean ± SD (range) or n (%)
Maternal age (y)	32.63 ± 4.76 (21.00–47.00)
≥35y	137 (31.4 %)
<35y	299 (68.6 %)
BMI (kg/m^2)	22.16 ± 3.14 (14.90–36.70)
Underweight (BMI <18.5)	36 (8.3 %)
Normal (18.5 ≤ BMI <24)	293 (67.2 %)
Overweight (BMI ≥24)	107 (24.5 %)
Duration of infertility (y)	4.21 ± 3.01 (1.00–17.00)
Infertility type	
Primary infertility[a]	200 (45.9 %)
Second infertility[b]	236 (54.1 %)
Diagnosis of infertility	
Female factor	228 (52.3 %)
Male factor	61 (14.0 %)
Both	134 (30.7 %)
Unexplained	13 (3.0 %)
Basal hormone profiles	
FSH (IU/L)	7.27 ± 2.95 (1.05–30.02)
LH (IU/L)	6.27 ± 6.92 (1.02–107.2)
AMH (ng/ml)	3.39 ± 2.93 (0.04–19.1)
Estradiol (pg/ml)	48.66 ± 51.05 (5.00–637.00)
AFC	12.97 ± 7.79 (0.00–42.00)
Oocyte insemination technique	
IVF	257 (71.6 %)
ICSI	102 (28.4 %)
Treatment protocol	
Long GnRH agonist protocol	112 (31.2 %)
GnRH antagonist protocol	61 (17.0 %)
Artificial cycle	134 (37.3 %)
Natural cycle	42 (11.7 %)
Mild stimulation	9 (2.5 %)
Other protocol	1 (0.3 %)
Controlled ovarian hyperstimulation outcomes	
Total number of oocytes retrieved	12.24 ± 6.81 (1.00–40.00)
Mature (MII) oocytes retrieved	8.48 ± 5.38 (0.00–25.00)
Normal (2 PN) fertilized oocytes	7.44 ± 4.93 (0.00–24.00)
Total embryos (n)	5.77 ± 4.19 (1.00–24.00)
High-quality embryo (n)	4.76 ± 3.76 (0.00–24.00)
Embryo transfer day	
Day 3	206 (57.4 %)
Day 5	133 (37.0 %)
Day 6	20 (5.1 %)
Number of embryos transferred	
1 embryo	173 (48.2 %)
2 embryos	186 (51.8 %)
Pregnancy outcomes	
Biochemical pregnancy[c]	190 (52.9 %)
Clinical pregnancy[d]	176 (49.0 %)
Miscarriage[e]	55 (28.9 %)
Live birth[f]	135 (37.6 %)

Note: SD, standard deviation; BMI, body mass index; AMH, anti-Mullerian hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; AFC, antral follicle count; GnRH, gonadotropin-releasing hormone; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

[a] Primary infertility was defined as the inability to conceive after at least one year of unprotected intercourse for women under 35 years old, or after six months for women 35 years and older.

[b] Secondary infertility was defined as the inability to conceive or carry a pregnancy to term following the birth of one or more biological children.

[c] Biochemical pregnancy was defined as a positive pregnancy test (serum β-hCG level>5 mIU/mL) 12 days following blastocyst transplantation or 14 days following cleavage-stage embryo transplantation.

[d] Clinical pregnancy was defined as the presence of a gestational sac with fetal heartbeat detected through ultrasound 3 weeks after embryo transfer.

[e] Miscarriage was defined as the termination of a pregnancy before 28 weeks of gestation with a fetal body weight of less than 1000 g.

[f] Live birth was defined as any birth event in which at least one baby was born alive on or after 28 weeks gestation.

2.5. Sample extraction and instrument analysis

The concentrations of 11 parent NEOs - ACE, THX, CLO, IMI, imidaclothiz (IMZ), DIN, flonicamid (FLO), sulfoxaflor (SUF), NIT, THI, and thiacloprid-amide (TA) - along with 4 primary metabolites [N-desmethyl-acetamiprid (N-dm-ACE), olefin-imidacloprid (OF-

Table 2
NEOs concentrations (ng/mL) in paired follicular fluid-serum samples (n = 436).

NEOs	Detection rate ≥ LOD (%)	GM ± SD	Percentile					
			Min	25th	50th	75th	95th	Max
			Follicular fluid					
N-dm-ACE	100	0.221 ± 0.590	0.024	0.111	0.208	0.416	1.360	6.994
IMI	42.56	0.048 ± 0.121	< LOD	< LOD	< LOD	0.08	0.263	1.630
CLO	41.42	0.035 ± 0.225	< LOD	< LOD	< LOD	0.049	0.215	3.376
ACE	25.40	0.005 ± 0.139	< LOD	< LOD	< LOD	< LOD	0.072	1.921
THX	24.49	0.056 ± 0.230	< LOD	< LOD	< LOD	< LOD	0.199	2.660
OF-IMI	4.81	0.071 ± 0.022	< LOD	< LOD	< LOD	< LOD	< LOD	0.366
TA	4.58	0.013 ± 0.013	< LOD	< LOD	< LOD	< LOD	< LOD	0.241
N-DMT	3.89	0.043 ± 0.004	< LOD	< LOD	< LOD	< LOD	< LOD	0.085
THI	3.89	0.003 ± 0.023	< LOD	< LOD	< LOD	< LOD	< LOD	0.406
IMZ	2.29	0.026 ± 0.090	< LOD	< LOD	< LOD	< LOD	< LOD	1.800
SUF	2.06	0.022 ± 0.010	< LOD	< LOD	< LOD	< LOD	< LOD	0.223
DIN	1.37	0.163 ± 0.024	< LOD	< LOD	< LOD	< LOD	< LOD	0.534
NIT	1.37	0.038 ± 0.297	< LOD	< LOD	< LOD	< LOD	< LOD	6.247
FLO	1.37	0.029 ± 0.167	< LOD	< LOD	< LOD	< LOD	< LOD	3.517
6-CN	0.23	0.196 ± 0.342	< LOD	< LOD	< LOD	< LOD	< LOD	0.909
			Serum					
N-dm-ACE	100	0.228 ± 0.548	0.021	0.116	0.207	0.411	1.246	5.078
IMI	19.91	0.051 ± 0.093	< LOD	< LOD	< LOD	< LOD	0.246	0.786
CLO	14.19	0.061 ± 0.107	< LOD	< LOD	< LOD	< LOD	0.155	1.240
ACE	13.73	0.020 ± 0.110	< LOD	< LOD	< LOD	< LOD	0.070	1.534
THX	20.37	0.058 ± 0.148	< LOD	< LOD	< LOD	< LOD	0.189	2.517
OF-IMI	9.38	0.039 ± 0.023	< LOD	< LOD	< LOD	< LOD	0.082	0.258
TA	0	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
N-DMT	0.23	0.148 ± 0.009	< LOD	< LOD	< LOD	< LOD	< LOD	0.334
THI	0.23	0.022 ± 0.001	< LOD	< LOD	< LOD	< LOD	< LOD	0.031
IMZ	0	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
SUF	2.75	0.044 ± 0.015	< LOD	< LOD	< LOD	< LOD	< LOD	0.196
DIN	0.46	0.294 ± 0.032	< LOD	< LOD	< LOD	< LOD	< LOD	0.944
NIT	4.35	0.018 ± 0.005	< LOD	< LOD	< LOD	< LOD	< LOD	0.093
FLO	0.23	0.017 ± 0.001	< LOD	< LOD	< LOD	< LOD	< LOD	0.024
6-CN	4.12	0.188 ± 0.067	< LOD	< LOD	< LOD	< LOD	< LOD	0.942

Note: NEOs, Neonicotinoids; GM, geometric mean; SD, standard deviation; LOD, limit of detection; Min, minimum value; Max, maximum value.

IMI), 6-chloronicotinic acid (6-CN), and N-desmethyl-thiamethoxam (N-DMT)] were quantified. To achieve this, a 250 μL sample of follicular fluid was fortified with 2.5 ng (equivalent to 50 μL from a 50 ng/mL stock solution) of each stable isotopically labeled internal standard in a 15 mL polypropylene (PP) tube. Following this, 2.5 mL of acetonitrile and 0.2 mL of saturated sodium chloride solution were added to each sample for matrix elimination from the follicular fluid. The samples were then vortexed, subjected to 10 min of ultrasonic extraction, and centrifuged at 4000 rpm for 5 min. The resulting supernatant was transferred to a fresh 15 mL PP tube and concentrated nearly to dryness using a gentle nitrogen stream from Organomation Associates Inc. (West Berlin, MA, USA). Subsequently, the extract was reconstituted with 250 μL of methanol/water (1:1, v/v) for subsequent instrumental analysis. The levels of the compounds were analyzed in both serum and follicular fluid utilizing a salting-out assisted liquid-liquid extraction (SALLE) technique and liquid chromatography-tandem mass spectrometry (LC-MS/MS), as detailed in our previous study [47]. The analysis of the target analytes was carried out using high-performance liquid chromatography equipment from Shimadzu (Kyoto, Japan) equipped with a BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters; Milford, MA, USA). For detection and quantification, a Triple QuadTM 5500 mass spectrometer from AB Sciex (Framingham, MA, USA) was employed. The analysis utilized either positive or negative electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. A gradient flow system employing water (A) and methanol (B) as mobile phases was utilized for the analysis process.

2.6. Quality assurance and quality control

To ensure accuracy and reliability, a procedure blank was created by following the complete extraction protocol without introducing any sample, consisting of methanol and Milli-Q water in a 1:1 ratio (v/v). Additionally, a midpoint calibration standard at 10 ng/mL was analyzed after every set of 10 samples to monitor any instrumental drift in response factors that may occur over time. Furthermore, pure solvent (methanol) injections were performed after every 10 samples to assess potential carryover of the target analytes between samples.

The limits of detection (LODs) and limits of quantification (LOQs) were established by analyzing follicular fluid or serum spiked with low levels of standards and isotope-labeled standards. The LODs and LOQs were defined as 3 times the signal-to-noise ratio (S/N) and 10 times the S/N, respectively. Here, the S/N was determined from replicate measurements of low-level spiked standards in both follicular fluid and serum. The LOD and LOQ values for each analyte are detailed in Table S6. For concentrations below the LOD, a value of LOD/$\sqrt{2}$ was assigned.

Table 3
Associations between follicular fluid and serum NEOs exposure and intermediate IVF/ICSI outcomes.

Exposure	Retrieved oocytes β (95 % CI)		Matured oocytes β (95 % CI)		2 PN zygotes β (95 % CI)		Good-quality embryos β (95 % CI)	
	Unadjusted	Adjusted[e]	Unadjusted	Adjusted[e]	Unadjusted	Adjusted[d]	Unadjusted	Adjusted[d]
				Follicular fluid				
THX	−0.04 (-0.11,0.02)	−0.04 (-0.11,0.02)	**−0.18 (-0.27,-0.10)[c]**	**−0.18 (-0.27,-0.10)[c]**	**−0.20 (-0.30,-0.11)[c]**	**−0.20 (-0.30,-0.11)[c]**	**−0.24(-0.36,-0.12)[c]**	**−0.24 (-0.36,-0.12)[c]**
IMI	**−0.29 (-0.34,-0.23)[c]**	**−0.29 (-0.35,-0.23)[c]**	**−0.20 (-0.28,-0.13)[c]**	**−0.20 (-0.28,-0.13)[c]**	**−0.24 (-0.32,-0.16)[c]**	**−0.24 (-0.32,-0.16)[c]**	**−0.25(-0.35,-0.16)[c]**	**−0.25 (-0.35,-0.16)[c]**
ACE	**−0.24 (-0.34,-0.14)[c]**	**−0.24 (-0.33,-0.14)[c]**	**−0.33 (-0.45,-0.21)[c]**	**−0.33 (-0.45,-0.21)[c]**	**−0.31 (-0.44,-0.18)[c]**	**−0.31 (-0.44,-0.18)[c]**	**−0.24(-0.40,-0.09)[b]**	**−0.24 (-0.40,-0.09)[b]**
CLO	**0.07 (0.01,0.13)[a]**	**0.07 (0.01,0.13)[a]**	0.01 (-0.06,0.08)	0.01 (-0.06,0.08)	0.02 (-0.05,0.10)	0.02 (-0.05,0.10)	0.02 (-0.08,0.11)	0.02 (-0.08,0.11)
Number of exposures	**−0.06 (-0.08,-0.04)[c]**	**−0.05 (-0.07,-0.03)[c]**	**−0.07 (-0.10,-0.05)[c]**	**−0.07 (-0.10,-0.05)[c]**	**−0.08 (-0.11,-0.05)[c]**	**−0.08 (-0.11,-0.05)[c]**	**−0.12(-0.16,-0.07)[c]**	**−0.07 (-0.11,-0.04)[c]**
Exposed or not	**0.14 (0.08,0.20)[c]**	**0.14 (0.08,0.20)[c]**	**0.12 (0.05,0.20)[c]**	**0.12 (0.05,0.20)[c]**	**0.14 (0.06,0.22)[c]**	**0.14 (0.06,0.22)[c]**	**0.16 (0.04,0.29)[a]**	**0.10 (0.00,0.20)[a]**
Serum								
THX	0.05 (-0.02,0.12)	0.05 (-0.02,0.12)	0.07 (-0.01,0.16)	0.07 (-0.01,0.16)	0.04 (-0.05,0.13)	0.04 (-0.05,0.13)	−0.01 (-0.13,0.11)	−0.01 (-0.13,0.11)
IMI	−0.01 (-0.08,0.06)	−0.01 (-0.09,0.06)	0.03 (-0.06,0.12)	0.03 (-0.06,0.12)	0.02 (-0.07,0.11)	0.02 (-0.07,0.11)	−0.06 (-0.18,0.06)	−0.06 (-0.18,0.06)
Number of exposures	0.01 (-0.02,0.04)	0.02 (-0.01,0.05)	−0.01 (-0.04,0.03)	−0.01 (-0.04,0.03)	−0.02 (-0.06,0.01)	−0.02 (-0.06,0.01)	**−0.06 (-0.12,0.00)[a]**	−0.04 (-0.09,0.00)
Exposed or not	0.02 (-0.04,0.07)	0.02 (-0.04,0.07)	0.03 (-0.04,0.10)	0.03 (-0.04,0.10)	0.05 (-0.02,0.13)	0.05 (-0.02,0.13)	**0.15 (0.03,0.27)[a]**	0.08 (-0.01,0.17)

Exposure	Oocytes mature rate β (95 % CI)		Fertilization rate β (95 % CI)		Cleavage rate β (95 % CI)		Good-quality embryo rate β (95 % CI)	
	Unadjusted	Adjusted[e]	Unadjusted	Adjusted[d]	Unadjusted	Adjusted[d]	Unadjusted	Adjusted[d]
				Follicular fluid				
THX	**−0.49(-0.63,-0.35)[c]**	**−0.50(-0.64,-0.36)[c]**	−0.16 (-0.40,0.08)	−0.18 (-0.44,0.07)	−0.19 (-0.78,0.40)	−0.20 (-0.79,0.39)	−0.09 (-0.28,0.10)	−0.07 (-0.26,0.12)
IMI	**0.14 (0.01,0.26)[a]**	0.12 (-0.01,0.25)	−0.17 (-0.37,0.04)	**−0.24 (-0.46,-0.02)[a]**	−0.02 (-0.54,0.50)	−0.03 (-0.55,0.49)	−0.03 (-0.19,0.13)	−0.06 (-0.22,0.10)
ACE	−0.16 (-0.36,0.04)	−0.17 (-0.37,0.03)	0.08 (-0.28,0.43)	0.19 (-0.18,0.57)	1.18 (-0.23,2.59)	1.24 (-0.17,2.66)	0.16 (-0.11,0.42)	0.20 (-0.07,0.47)
CLO	**−0.30(-0.42,-0.18)[c]**	**−0.30(-0.42,-0.18)[c]**	**0.22 (0.01,0.43)[a]**	0.04 (-0.18,0.26)	0.09 (-0.42,0.59)	0.03 (-0.49,0.54)	0.00 (-0.16,0.15)	0.01 (-0.15,0.17)
Number of exposures	**−0.06(-0.11,-0.02)[b]**	**−0.06(-0.11,-0.02)[b]**	−0.02 (-0.10,0.05)	−0.04 (-0.12,0.04)	0.02 (-0.17,0.20)	0.01 (-0.17,0.20)	0.01 (-0.05,0.07)	0.02 (-0.04,0.07)
Exposed or not	0.07 (-0.06,0.20)	0.07 (-0.06,0.20)	−0.05 (-0.26,0.16)	0.14 (-0.08,0.37)	−0.44 (-0.94,0.05)	−0.41 (-0.91,0.10)	−0.10 (-0.26,0.06)	−0.10 (-0.26,0.06)
				Serum				
THX	0.03 (-0.12,0.18)	0.03 (-0.12,0.17)	**−0.24 (-0.48,-0.01)[a]**	**−0.28 (-0.53,-0.03)[a]**	**−0.65 (-1.18,-0.12)[a]**	**−0.67 (-1.20,-0.14)[a]**	−0.13 (-0.31,0.06)	−0.13 (-0.32,0.06)
IMI	0.05 (-0.10,0.20)	0.04 (-0.12,0.19)	0.15 (-0.12,0.41)	0.04 (-0.23,0.32)	−0.02 (-0.64,0.59)	−0.04 (-0.66,0.58)	**−0.20 (-0.39,-0.01)[a]**	**−0.22 (-0.41,-0.03)[a]**
Number of exposures	**−0.06 (-0.12,0.00)[a]**	**−0.06 (-0.12,0.00)[a]**	**−0.15 (-0.24,-0.06)[b]**	**−0.14 (-0.24,-0.05)[b]**	**−0.30 (-0.51,-0.08)[b]**	**−0.30 (-0.51,-0.08)[b]**	−0.05 (-0.13,0.02)	−0.05 (-0.12,0.03)
Exposed or not	0.04 (-0.08,0.16)	0.05 (-0.08,0.17)	**0.22 (0.01,0.42)[a]**	**0.24 (0.03,0.46)[a]**	0.11 (-0.38,0.61)	0.12 (-0.38,0.62)	0.07 (-0.08,0.23)	0.06 (-0.09,0.22)

Note: NEOs, Neonicotinoids; β, regression coefficient; CI, confidence interval; BMI, body mass index; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

[a] $p < 0.05$.

[b] $p < 0.01$.

[c] $p < 0.001$.

[d] Models adjusted for women's age, BMI and oocyte insemination technique.

[e] Models adjusted for women's age and BMI.

Table 4
Associations between follicular fluid and serum NEOs exposure and IVF/ICSI clinical outcomes.

Exposure	Biochemical pregnancy OR (95 % CI)		Clinical pregnancy OR (95 % CI)		Abortion OR (95 % CI)		Live birth OR (95 % CI)	
	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]
Follicular fluid								
THX	1.039 (0.634,1.703)	1.062 (0.644,1.752)	0.987 (0.603,1.616)	0.985 (0.598,1.622)	1.313 (0.706,2.443)	1.347 (0.720,2.520)	0.883 (0.528,1.475)	0.901 (0.534,1.519)
IMI	**0.490 (0.319,0.752)** *	**0.497 (0.322,0.767)** **	**0.480 (0.312,0.739)** **	**0.488 (0.316,0.755)** **	0.903 (0.516,1.578)	0.884 (0.503,1.552)	**0.486 (0.309,0.764)** **	**0.493 (0.311,0.782)** **
ACE	0.859 (0.525,1.407)	0.919 (0.557,1.517)	0.927 (0.566,1.518)	0.982 (0.595,1.622)	0.670 (0.332,1.354)	0.664 (0.326,1.351)	1.011 (0.608,1.681)	1.101 (0.654,1.854)
CLO	1.173 (0.771,1.784)	1.230 (0.801,1.888)	1.227 (0.808,1.865)	1.259 (0.821,1.931)	1.167 (0.676,2.015)	1.163 (0.668,2.025)	1.004 (0.652,1.546)	1.074 (0.688,1.675)
Number of exposures	0.935 (0.808,1.080)	0.955 (0.824,1.106)	0.941 (0.814,1.088)	0.953 (0.822,1.105)	1.003 (0.830,1.213)	1.007 (0.830,1.222)	0.921 (0.791,1.073)	0.944 (0.808,1.102)
Exposed or not	1.135 (0.721,1.788)	1.126 (0.711,1.784)	0.925 (0.588,1.455)	0.932 (0.588,1.475)	1.021 (0.564,1.847)	1.029 (0.566,1.874)	1.105 (0.694,1.758)	1.083 (0.674,1.741)
Serum								
THX	0.895 (0.532,1.505)	0.932 (0.551,1.576)	0.945 (0.562,1.589)	0.992 (0.586,1.679)	0.832 (0.409,1.692)	0.804 (0.393,1.643)	0.949 (0.554,1.626)	1.017 (0.587,1.761)
IMI	1.001 (0.590,1.698)	0.991 (0.579,1.697)	0.976 (0.575,1.655)	0.957 (0.559,1.637)	0.887 (0.435,1.807)	0.850 (0.414,1.747)	1.116 (0.650,1.916)	1.131 (0.649,1.970)
Number of exposures	1.002 (0.813,1.235)	1.011 (0.818,1.249)	0.955 (0.775,1.177)	0.955 (0.773,1.179)	0.923 (0.696,1.223)	0.924 (0.695,1.228)	1.042 (0.841,1.292)	1.055 (0.848,1.312)
Exposed or not	1.122 (0.740,1.699)	1.063 (0.698,1.620)	1.204 (0.795,1.823)	1.155 (0.758,1.759)	1.027 (0.596,1.770)	1.035 (0.597,1.794)	1.124 (0.733,1.723)	1.048 (0.677,1.623)

Note: NEOs, Neonicotinoids; OR, odds ratio; CI, confidence interval; BMI, body mass index; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[a] Models adjusted for women's age, BMI and oocyte insemination technique.

2.7. Statistical analyses

Statistical analysis was conducted utilizing R version 4.3.2 (R Foundation for Statistical Computing, Austria). Descriptive statistics were employed to summarize demographic characteristics, clinical outcomes, and concentrations of NEOs. Demographic characteristics were expressed as the mean ± standard deviation (SD) or as numbers with corresponding percentages where applicable. The distribution and detection rates of NEOs in both follicular fluid and serum samples were also presented.

NEOs with a detection rate exceeding 15 % were chosen for analysis to ensure statistical robustness. For N-dm-ACE, which had a detection rate of 100 %, women were divided into tertiles based on their follicular fluid and serum N-dm-ACE levels. Linear trend tests across increasing N-dm-ACE exposure groups were performed by treating the tertiles as ordinal variables (1, 2, 3) in the linear regression model. Multivariate generalized linear models were employed to gauge the relationships between NEO concentrations or the number of NEO exposures and IVF/ICSI outcomes. The association estimates were summarized using beta (β) coefficients and odds ratios (ORs) along with their corresponding 95 % confidence intervals (CIs). Potential confounding factors were selected based on prior studies concerning the impacts of environmental pollutant exposure on IVF/ISCI outcomes [48]. Height and weight measurements taken at the initiation of the IVF/ISCI cycle were utilized to compute body mass index (BMI) (kg/m^2), which was categorized according to Chinese population standards as underweight (BMI <18.5 kg/m^2), normal weight ($18.5 \leq$ BMI <24 kg/m^2), or overweight (BMI ≥ 24.0 kg/m^2) [49]. A woman's age was determined based on her date of birth. The models incorporated the following covariates: age (a continuous variable) and BMI (a categorical variable). Besides the number of retrieved oocytes and matured oocytes, and the oocyte maturation rate, other outcomes included oocyte fertilization technology as a covariate. As only data from the initial embryo transfer were utilized, the potential clustering effect of multiple cycles per woman was not considered.

To ensure the reliability of the results, sensitivity analyses were carried out. A stratified analysis was conducted based on an age cutoff of 35 years to examine the potential influence of age on the associations observed. Furthermore, after excluding other infertility factors, a reevaluation was performed to explore the relationship between NEO exposure and IVF/ICSI outcomes, aiming to determine the impact of additional infertility factors on the consistency of the results. Additionally, NEO concentration was included as a continuous variable in the model to reassess its effect on IVF/ICSI outcomes. Overall, the stratified and sensitivity analyses confirmed the stability and biological plausibility of our findings. Statistical significance was defined as $P < 0.05$.

3. Results

The current study included a total of 436 women, and relevant demographic characteristics, female basal hormone profiles, ovarian stimulation protocols, and clinical outcomes of IVF/ICSI are detailed in Table 1. The participants were predominantly nulliparous, with a mean age of 32.63 years (standard deviation = 4.76) and a body mass index (BMI) of 22.16 kg/m2 (standard deviation = 3.14). In terms of pregnancy history, 54.1 % of the women had secondary infertility, while 45.9 % experienced primary infertility. The causes

Table 5
Associations between follicular fluid and serum N-dm-ACE concentrations and intermediate IVF/ICSI outcomes.

N-dm-ACE (ng/mL)	Retrieved oocytes β (95 % CI)		Matured oocytes β (95 % CI)		2 PN zygotes β (95 % CI)		Good-quality embryos β (95 % CI)	
	Unadjusted	Adjusted[e]	Unadjusted	Adjusted[e]	Unadjusted	Adjusted[d]	Unadjusted	Adjusted[d]
Follicular fluid								
T1 (<0.138)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
T2 (0.138–0.320)	**−0.14 (-0.21,-0.07)[c]**	**−0.11 (-0.18,-0.04)[b]**	**−0.24 (-0.32,-0.15)[c]**	**−0.18 (-0.27,-0.09)[c]**	**−0.22 (-0.31,-0.12)[c]**	**−0.19 (-0.28,-0.10)[c]**	**−0.14 (-0.26,-0.03)[a]**	**−0.12 (-0.24,0.00)[a]**
T3 (>0.320)	**−0.08 (-0.15,-0.01)[c]**	−0.04 (-0.11,0.02)	**−0.14 (-0.22,-0.06)[c]**	**−0.10 (-0.19,-0.02)[c]**	**−0.14 (-0.23,-0.05)[c]**	**−0.12 (-0.21,-0.03)[c]**	−0.11 (-0.22,0.00)	−0.09 (-0.20,0.02)
P-trend	**0.02**	0.19	**0.001**	**0.01**	**0.002**	**0.01**	0.06	0.12
Serum								
T1 (<0.142)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
T2 (0.142–0.321)	0.02 (-0.05,0.08)	0.01 (-0.06,0.07)	0.01 (-0.07,0.10)	0.02 (-0.06,0.11)	0.04 (-0.05,0.13)	0.04 (-0.05,0.13)	−0.01 (-0.12,0.10)	−0.01 (-0.13,0.10)
T3 (>0.321)	−0.05 (-0.12,0.02)	−0.03 (-0.10,0.04)	−0.08 (-0.17,0.00)	−0.06 (-0.14,0.03)	**−0.10 (-0.19,-0.01)[a]**	−0.08 (-0.17,0.01)	−0.11 (-0.23,0.01)	−0.09 (-0.21,0.02)
P-trend	0.15	0.47	0.06	0.20	**0.04**	0.09	0.06	0.12

N-dm-ACE (ng/mL)	Oocytes mature rate β (95 % CI)		Fertilization rate β (95 % CI)		Cleavage rate β (95 % CI)		Good-quality embryo rate β (95 % CI)	
	Unadjusted	Adjusted[e]	Unadjusted	Adjusted[d]	Unadjusted	Adjusted[d]	Unadjusted	Adjusted[d]
Follicular fluid								
T1 (<0.138)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
T2 (0.138–0.320)	**−0.26 (-0.41,-0.11)[c]**	**−0.25 (-0.40,-0.10)[b]**	0.08 (-0.17,0.33)	−0.15 (-0.42,0.12)	0.03 (-0.58,0.64)	−0.07 (-0.69,0.55)	**0.19 (0.00,0.38)[a]**	**0.20 (0.01,0.39)[a]**
T3 (>0.320)	**−0.16 (-0.30,-0.01)[c]**	**−0.18 (-0.32,-0.03)[a]**	−0.01 (-0.25,0.24)	−0.10 (-0.35,0.16)	0.01 (-0.58,0.60)	−0.01 (-0.61,0.59)	0.08 (-0.11,0.26)	0.05 (-0.13,0.24)
P-trend	**0.03**	**0.02**	0.99	0.43	0.97	0.96	0.38	0.52
Serum								
T1 (<0.142)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
T2 (0.142–0.321)	0.02 (-0.13,0.16)	0.02 (-0.13,0.17)	0.19 (-0.07,0.44)	0.11 (-0.16,0.37)	0.05 (-0.55,0.65)	0.02 (-0.58,0.62)	−0.13 (-0.31,0.06)	−0.13 (-0.32,0.05)
T3 (>0.321)	−0.03 (-0.18,0.12)	−0.05 (-0.20,0.10)	−0.24 (-0.48,0.01)	**−0.36 (-0.62,-0.10)[b]**	0.01 (-0.61,0.63)	−0.03 (-0.66,0.60)	−0.03 (-0.22,0.16)	−0.04 (-0.24,0.15)
P-trend	0.73	0.54	0.05	**0.01**	0.98	0.93	0.75	0.63

Note: NEOs, Neonicotinoids; β, regression coefficient; CI, confidence interval; BMI, body mass index; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.
[d] Models adjusted for women's age, BMI and oocyte insemination technique.
[e] Models adjusted for women's age and BMI.

of infertility among the women undergoing IVF/ICSI were attributed to male factors (14.0 %), female factors (52.3 %), combined factors (30.7 %), and unexplained infertility (3.0 %). The long gonadotropin-releasing hormone (GnRH) agonist (31.2 %) and GnRH antagonist (17.0 %) ovarian stimulation protocols were the most commonly employed controlled ovarian hyperstimulation (COH) protocols in the study cohort.

Out of the 436 women included in the study, 359 underwent at least one embryo transfer. Among these 359 transplantation cycles, 102 (28.4 %) utilized ICSI, while the remaining 257 (71.6 %) involved IVF. The range of total oocytes retrieved varied from 1 to 40, with a mean ± standard deviation of 12.24 ± 6.81. On average, 7.44 ± 4.93 oocytes were successfully fertilized (2 PN zygotes) and developed into 4.76 ± 3.76 good-quality embryos. The distribution consisted of 179 fresh transfers and 180 frozen transfers, with 51.8 % involving two embryo transfers. Furthermore, 57.4 % of the transfers took place on day 3 at the cleavage stage. The percentages of cycles that underwent embryo transfer resulting in biochemical pregnancy, clinical pregnancy, and live birth were 52.9 %, 49.0 %, and 37.6 %, respectively.

The concentrations of NEOs and their metabolites in follicular fluid and serum samples are presented as GM ± SD and percentiles (25th, 50th, 75th, and 95th) in Table 2. The GM follicular fluid concentrations of the target analytes varied from 0.003 (THI) to 0.221 (N-dm-ACE) ng/mL in women with infertility, while the concentration range in serum spanned from 0.017 (FLO) to 0.294 (DIN) ng/mL. Among the 15 target analytes, N-dm-ACE was detected in all follicular fluid and serum samples. In follicular fluid, IMI, CLO, ACE,

Table 6
Associations between follicular fluid and serum N-dm-ACE concentrations and IVF/ICSI clinical outcomes.

N-dm-ACE (ng/mL)	Biochemical Pregnancy		Clinical Pregnancy		Abortion		Live birth	
	OR (95 % CI)		OR (95 % CI)		OR (95 % CI)		OR (95 % CI)	
	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]
Follicular fluid								
T1 (<0.138)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
T2 (0.138–0.320)	0.76 (0.46,1.27)	0.79 (0.47,1.33)	0.74 (0.44,1.23)	0.78 (0.47,1.31)	1.22 (0.64,2.32)	1.15 (0.60,2.21)	0.64 (0.38,1.08)	0.70 (0.41,1.19)
T3 (>0.320)	**0.58 (0.35,0.97)***	0.61 (0.36,1.02)	0.62 (0.37,1.04)	0.66 (0.39,1.10)	0.78 (0.39,1.56)	0.76 (0.37,1.53)	**0.59 (0.35,0.99)***	0.62 (0.37,1.07)
P-trend	**0.04**	0.06	0.07	0.11	0.50	0.45	0.05	0.08
Serum								
T1 (<0.142)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
T2 (0.142–0.321)	1.07 (0.64,1.77)	1.03 (0.61,1.72)	1.03 (0.62,1.71)	1.00 (0.60,1.68)	1.25 (0.63,2.47)	1.22 (0.61,2.43)	0.94 (0.56,1.58)	0.91 (0.54,1.54)
T3 (>0.321)	0.85 (0.51,1.41)	0.87 (0.52,1.47)	0.82 (0.49,1.36)	0.82 (0.49,1.38)	1.34 (0.68,2.65)	1.33 (0.67,2.64)	0.75 (0.44,1.27)	0.79 (0.46,1.35)
P-trend	0.52	0.61	0.44	0.46	0.39	0.42	0.28	0.38

Note: NEOs, Neonicotinoids; OR, odds ratio; CI, confidence interval; BMI, body mass index; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

[a] Models adjusted for women's age, BMI and oocyte insemination technique.

and THX exhibited detection rates ranging from 24.5 % to 42.6 %. Conversely, the detection rates of THI, N-DMT, TA, IMZ, DIN, FLO, NIT, 6-CN, SUF, and OF-IMI were lower (<15 %) (Table 2). Additionally, THX and IMI were identified in more than 15 % of the serum samples, while other NEOs had lower detection rates (<15 %). TA and IMZ were undetectable in any serum sample, although they were present in some paired follicular fluid samples. Among the 136 women, only one type of NEO was detected in the follicular fluid of one individual, whereas nine types of NEOs were detected in two women. Moreover, 68.8 % of women exhibited detection of two or more types of NEOs in their follicular fluid. In serum samples, the minimum number of NEOs detected in women was one type, observed in 200 individuals, while the maximum exposure involved six types, found in two individuals. Notably, 54.1 % of women had two or more NEOs detected in their serum samples.

The associations between follicular fluid and serum NEO levels and IVF/ICSI outcomes are summarized in Tables 3 and 4 for intermediate and clinical outcomes, respectively. Our findings suggest that exposure to NEOs is linked to poor intermediate outcomes. Women with detectable levels of THX, IMI, and ACE in follicular fluid exhibited a reduction in 2 PN zygotes and good-quality embryos compared to those without detectable levels, as per multivariate generalized linear models adjusting for age, BMI, and oocyte insemination technique ($P < 0.01$). Additionally, compared to women without detectable levels of corresponding NEOs in follicular fluid, those with detectable levels of IMI, ACE, and CLO experienced a significant decrease in the number of retrieved oocytes ($P < 0.05$). Moreover, women with detectable levels of THX, IMI, and ACE in their follicular fluid demonstrated a significant reduction in the number of mature oocytes ($P < 0.001$). The rate of oocyte maturation was lowered in women with detectable levels of THX and CLO in follicular fluid ($P < 0.001$), while the fertilization rate decreased in those with detectable levels of IMI ($P < 0.05$). Furthermore, women with detectable levels of THX in serum displayed lower fertilization and cleavage rates ($P < 0.05$), and those with detectable levels of IMI showed a diminished rate of high-quality embryos ($P < 0.05$) (Table 3).

In our investigation of associations between NEO exposure and clinical outcomes in IVF/ICSI procedures, we found noteworthy results. Women with detectable levels of IMI in follicular fluid had lower odds of achieving biochemical pregnancy (adjusted odds ratio [OR] = 0.497; 95 % confidence interval [CI]: 0.322, 0.767), clinical pregnancy (adjusted OR = 0.488; 95 % CI: 0.316, 0.755), and live birth (adjusted OR = 0.493; 95 % CI: 0.311, 0.782) compared to those without IMI detected in their follicular fluid ($P < 0.01$) (Table 4). Conversely, our analysis did not reveal any significant associations between serum NEO concentrations and biochemical pregnancy, clinical pregnancy, miscarriage, or live birth rate ($P > 0.05$) (Table 4). These findings shed light on the potential impact of NEO exposure on clinical outcomes in assisted reproductive technologies, particularly in relation to follicular fluid concentrations of these substances.

In order to investigate the potential dose-effect relationship between N-dm-ACE concentration in follicular fluid or serum and pregnancy outcomes, participants were divided into three groups based on their exposure levels using the tertile method. Both crude and multiple-adjusted models revealed statistically significant associations between high follicular fluid concentrations of N-dm-ACE and decreased numbers of mature oocytes (*P*-trend = 0.01) and 2 PN zygotes (*P*-trend = 0.01), as well as a lower oocyte maturity rate (*P*-trend = 0.02; Table 5). Moreover, a multivariate generalized linear model adjusted for age, BMI, and oocyte insemination technique demonstrated a significant association between high serum concentrations of N-dm-ACE and reduced fertilization rates (*P*-trend = 0.01; Table 5). However, no significant dose-effect relationships were observed between follicular fluid N-dm-ACE concentrations and biochemical pregnancy, clinical pregnancy, miscarriage rate, or live birth rate (*P*-trend>0.05). Similarly, no significant dose-effect relationship was found between serum N-dm-ACE levels and clinical outcomes (Table 6). These results provide insights into the potential impact of N-dm-ACE exposure on specific IVF/ICSI outcomes, highlighting the importance of considering both follicular fluid and serum concentrations in assessing reproductive health implications.

In addition to investigating the impact of individual NEO exposure on IVF/ICSI outcomes, we also explored the influence of the

number of NEO exposures in follicular fluid and serum on IVF/ICSI outcomes. Our findings indicated a significant decrease in the number of retrieved oocytes, mature oocytes, 2 PN zygotes, good-quality embryos, and oocyte maturity rate as the number of exposed NEOs increased in follicular fluid ($P < 0.05$) (Table 3). Furthermore, except for women with N-dm-ACE exposure, those with at least one NEO exposure exhibited a notable reduction in the number of retrieved oocytes, mature oocytes, 2 PN zygotes, and good-quality embryos compared to women without NEO exposure in follicular fluid ($P < 0.05$) (Table 3). As the number of NEOs in serum increased, a significant decrease was observed in the oocyte maturation rate, fertilization rate, and cleavage rate ($P < 0.05$) (Table 3). Similarly, except for N-dm-ACE, women with at least one type of NEO detected in their serum had a lower fertilization rate compared to women without any NEOs detected ($P < 0.05$) (Table 3). These results underscore the potential adverse effects of NEO exposure on various aspects of IVF/ICSI outcomes, emphasizing the importance of considering the cumulative impact of NEO exposure on reproductive health.

We conducted stratification analyses to examine the relationship between NEO exposure and clinical outcomes, stratified by age (<35 or ≥35 years). Interestingly, similar associations were observed in both groups of women, indicating consistent findings across different age brackets (Table S1). Furthermore, when analyzing the inverse associations of N-dm-ACE concentrations with clinical outcomes among younger women (<35 years) and older women (≥35 years), we found that the associations were stronger and displayed a more consistent monotonic trend in the younger age group (Table S3). To address potential confounding factors related to male infertility, we conducted subgroup analyses focusing on participants diagnosed with female factor infertility. Remarkably, similar results were observed in this subpopulation, reaffirming the robustness of the findings (Table S2 and Table S4). Additionally, we included NEO concentration as a continuous variable in the analysis model and found that the outcomes remained consistent, further supporting the validity of the associations observed (Table S5).

4. Discussion

To the best of our knowledge, our study represents the first investigation utilizing serum and follicular fluid samples to explore the link between environmental NEO exposure and pregnancy outcomes in women with infertility undergoing IVF/ICSI. Apart from IMZ and TA, other NEOs were detectable in serum and follicular fluid samples collected from women with infertility. Our findings suggest that NEO exposure may have detrimental effects on both intermediate and final outcomes of IVF/ICSI. Specifically, women with detectable levels of THX, IMI, and ACE in follicular fluid exhibited reduced numbers of mature oocytes, 2 PN zygotes, and good-quality embryos compared to those without detectable levels. Moreover, a significant negative correlation was observed between the number of exposed NEOs in follicular fluid and the quantities of total and mature oocytes, 2 PN zygotes, and good-quality embryos. Higher concentrations of N-dm-ACE in follicular fluid were associated with fewer mature oocytes and 2 PN zygotes, as well as a lower oocyte maturity rate. Women with detectable levels of THX in serum displayed lower fertilization and cleavage rates, while those with detectable levels of IMI had a reduced rate of high-quality embryos. Furthermore, compared to women without detectable levels of IMI in follicular fluid, those with detectable levels had decreased odds of biochemical pregnancy, clinical pregnancy, and live birth. However, exposure to other NEOs did not significantly impact clinical outcomes. In conclusion, our study offers additional evidence supporting the adverse effects of NEOs on reproduction, encompassing a wide array of pregnancy outcomes following exposure to various NEOs and their metabolites.

The global utilization of NEOs has been on the rise owing to their wide-ranging insecticidal properties, unique neurotoxicity patterns, and minimal toxicity in mammals. Extensive research has demonstrated that humans are commonly exposed to NEOs, leading to adverse effects on human health [50]. Wang et al.[51] conducted an analysis on 6 NEOs in n = 336 indoor dust samples from various Chinese cities, such as Taiyuan, Wuhan, and Shenzhen, revealing that ACE and IMI were detectable in 98.8 % and 99.7 % of the samples, respectively. In a study carried out in the United States, researchers identified four new nicotine insecticides—THX, CLO, IMI, and THI— in soil samples collected from agricultural areas [52]. Chen et al.Chen et al., 2020 [53] investigated the health risks associated with dietary exposure to NEOs in the Chinese adult population and found that IMI and ACE were the most commonly used neonics in China, with detection rates exceeding 50 %, along with an increasing usage of THI and CLO. These studies indicate that humans may come into contact with NEOs through air inhalation, dust ingestion, dermal absorption, and dietary consumption. Considering the widespread presence of NEOs in food samples and the necessity for additional research on toxicological thresholds in mammals, the potential risks of dietary exposure to total NEOs should not be underestimated.

Several studies have investigated the concentrations of NEOs in blood or urine samples collected from the general population. For instance, Zhang et al. [54] examined the levels of six types of NEOs (CLO, THM, IMI, DIN, ACE, and THD) in urine samples from 324 volunteers in China, revealing a detection rate exceeding 92 %, indicating widespread exposure to NEOs among the population. Similarly, Xu et al.[55] reported the presence of NEOs in whole blood samples from Chinese university students aged 20–27 years, with median concentrations ranging from 0.08 (THM) to 0.78 (OF-IMI) ng/mL. Comparatively, the concentrations of NEOs in most urine samples from previous studies were slightly higher than those detected in both the serum and follicular fluid samples analyzed in our study [24,55]. This difference may be attributed to the fact that a significant proportion of NEOs (19–55 %) are excreted by the kidneys within 24 h [21,22], resulting in higher NEO concentrations in urine samples than in serum and other sample types. The geometric mean concentrations of NEOs in serum from our study were found to be similar to those reported in studies on the serum levels of NEOs in the general population of Guangdong Province. However, with the exception of N-dm-ACE, the detection rate of other NEOs was lower than that reported in these studies [56,57]. This variance could be due to various factors such as sample handling and storage conditions, which may have influenced the degradation of NEOs in the follicular fluid and serum, potentially resulting in a reduced detection rate. Consequently, further research is warranted to better understand the factors influencing NEO detection rates in different sample types and populations. In a previous study conducted by our team [47], we utilized the same methodology to analyze

neonicotinoid insecticides (NEOs) in fresh follicular fluid and observed a higher detection rate compared to the current study. The existing research on the exposure of follicular fluid to NEOs is limited, highlighting the need for further investigations in this area. In our current study, we aimed to provide a quantitative assessment of NEOs and their metabolite concentrations in follicular fluid and serum as a means of objectively evaluating specific environmental NEO exposures in women before conception. Our findings revealed NEO detection rates ranging from 0.23 % to 100 % in follicular fluid and from 0 % to 100 % in serum. Overall, the burden of NEOs in follicular fluid was lower than that in serum, indicating a potential barrier effect on the transfer of NEOs from serum to follicular fluid. This observation underscores the importance of understanding the dynamics of NEO distribution in different biological compartments and its implications for human health.

Our research focused on investigating the associations between NEO exposure and intermediate and final outcomes of IVF/ICSI. Our study revealed that women with NEO exposure tended to have poorer intermediate IVF/ICSI outcomes, particularly in terms of the number of oocytes retrieved and embryo quality. Interestingly, we observed a negative correlation between the number of NEOs present in follicular fluid and the quantity of total oocytes, 2 PN zygotes, and high-quality embryos obtained. However, for the majority of NEOs analyzed, the impact on final pregnancy outcomes did not reach statistical significance. Notably, N-dm-ACE, which was detected in 100 % of both follicular fluid and serum samples, was stratified into tertile concentrations to explore the dose-response relationship between N-dm-ACE exposure and reproductive outcomes. Our results indicated that women with infertility who were exposed to higher levels of N-dm-ACE exhibited inferior intermediate IVF/ICSI outcomes. Furthermore, a dose-response pattern was observed between N-dm-ACE exposure levels and both reproductive and pregnancy outcomes. Importantly, the effects observed in follicular fluid samples were more pronounced compared to serum samples, suggesting that NEO exposure primarily impacts oocyte maturation and in vitro embryo development in women undergoing infertility treatment. Despite the negative impact on intermediate IVF/ICSI outcomes, our findings indicated that once successful embryo implantation occurred, NEO exposure did not significantly influence final reproductive outcomes. This suggests that while NEO exposure may affect early stages of assisted reproduction, it may not have a substantial effect on the overall success of pregnancy following embryo implantation.

Currently, research on the reproductive toxicity and mechanisms of NEOs in mammals primarily relies on animal models. Previous animal studies have demonstrated that exposure to IMI can result in damage to the female reproductive system. This damage is characterized by abnormalities in the morphology of reproductive organs, alterations in hormone expression levels, and decreased rates of embryonic development [34,58–60]. Studies by Ishikawa et al. [61] have shown that high concentrations of ACE exceeding 30 ppm and IMI exceeding 10 ppm can inhibit nuclear maturation of porcine oocytes. In rat ovaries exposed to IMI through oral administration, there is a significant increase in lipid peroxide (LPO) levels due to decreased levels of the antioxidant glutathione and reduced activities of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase [34]. Furthermore, research by Yazaki et al. [62] revealed that the nuclear maturation rate of porcine oocytes decreases with an increase in the LPO ratio. Ishikawa et al.Ishikawa et al., 2014 [63] also found that adding antioxidants to the medium during in vitro maturation of porcine oocytes significantly enhances nuclear maturation, indicating that oxidative stress has a substantial impact on porcine oocyte maturation. This suggests that the decline in nuclear maturation may be attributed to the influence of oxidative stress, as triglycerides are oxidized to LPO in oocytes upon direct exposure to IMI. While these studies provide valuable insights into the effects of NEOs on mammalian reproductive health, further animal experiments are necessary to gain a comprehensive understanding of the underlying mechanisms involved. Additional research will help uncover the intricate details of how NEO exposure affects reproductive health in mammals.

Our study possesses several notable strengths that contribute to the advancement of knowledge in this field. Firstly, to the best of our understanding, this research represents the first attempt to assess NEO exposure levels and comprehensively investigate pregnancy outcomes in a sizable cohort of Chinese women undergoing assisted conception. By utilizing assisted reproductive technology (ART), we were able to track pregnancy outcomes sequentially, from oocyte retrieval and fertilization to embryo quality, rates of fertilization and implantation, as well as clinical pregnancy and live birth. These detailed outcomes provide valuable insights that are not easily observable in naturally conceiving women. Secondly, our study is believed to be the pioneering investigation into the presence of NEOs and their primary metabolites in the follicular fluid of women experiencing infertility. The follicular fluid serves as a critical microenvironment where developing oocytes and their surrounding somatic cells interact directly, playing a crucial role in oocyte development. Compared to alternative sample types such as urine, the concentrations of NEOs and metabolites in follicular fluid can more accurately reflect the actual exposure levels of oocytes and the biologically effective doses targeting the organs. Lastly, we conducted thorough stratified and sensitivity analyses on our results, ensuring the robustness, stability, and biological plausibility of our findings. By systematically validating our outcomes through these analyses, we enhance the reliability and credibility of our research conclusions.

Despite its strengths, our study is not without limitations that warrant consideration. Firstly, the generalizability of our findings may be limited to the specific population of women undergoing assisted conception and may not extend to those who conceive naturally. Secondly, the presence of residual confounding factors cannot be entirely discounted, as we lacked data on certain covariates such as diet, physical activities, other environmental pollutants, and the potential exposure of the male partners. These unaccounted variables could potentially influence the outcomes observed in our study. Furthermore, while our results suggest a relationship between NEOs and their metabolites with assisted pregnancy outcomes in women with infertility, the exact mechanisms underlying this association remain unclear. To gain a deeper understanding of these relationships, further in vitro and *in vivo* experiments involving exposure to these metabolites are essential. Investigating the specific mechanisms by which NEOs impact reproductive outcomes will be crucial for elucidating the complex interplay between environmental exposures and fertility. Therefore, additional research in this field is imperative to provide more insights into the intricate interactions between environmental exposures, NEOs, and reproductive outcomes. By addressing these limitations and conducting further investigations, we can advance our understanding of how environmental factors influence fertility and assisted reproductive outcomes in women.

5. Conclusion

Our study offers compelling evidence that environmental exposure to NEOs before conception can have detrimental effects on clinical outcomes in IVF/ICSI pregnancies, particularly impacting intermediate reproductive outcomes such as total and maternal oocyte yield, fertilization rates, and embryo quality. While non-modifiable factors like patient age are well-known predictors of IVF/ICSI success, our research underscores the significance of identifying and addressing modifiable factors like avoiding NEO exposure. This study serves as a cornerstone for future investigations into the influence of NEO exposure levels in the ovarian microenvironment on reproductive function. By establishing reference values and opening up new research directions in reproductive toxicology, our findings pave the way for further exploration in this field. Subsequent studies should delve into the effects of pesticide mixture exposures and extend the investigation to assess the potential impacts of NEOs on male and couple reproductive health. To deepen our comprehension of the impact of NEOs on female fertility, future research endeavors, especially those incorporating large sample sizes and fundamental experimental studies, are crucial. By conducting comprehensive studies, we can enhance our understanding of the potential effects of NEOs on reproductive health and fertility outcomes, contributing to the advancement of knowledge in this critical area of research.

CRediT authorship contribution statement

Ziyu Liu: Data curation, Methodology, Writing – original draft. **Nijie Li:** Data curation, Formal analysis, Methodology. **Linan Xu:** Data curation, Resources, Software. **Rui Huang:** Data curation, Investigation, Validation. **Zhenhan Xu:** Data curation, Methodology, Software. **Guihua Liu:** Funding acquisition, Project administration, Supervision. **Xiaoyan Liang:** Conceptualization, Investigation, Supervision. **Xing Yang:** Funding acquisition, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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