Abstract

**Background:** Infertility remains a significant issue in the world of health. Now Assisted Reproductive Technology (ART) is widely used to help couples with infertility. In ART diminish ovarian reserve patients have a very low success rate of pregnancy. To help patients with Diminished Ovarian Reserve (DOR), research is conducted using an animal model. vinylcyclohexene diepoxide (VCD) has ovotoxic effects. This study looked at the use of VCD in inducing targeted DOR conditions in animals (rats), create a model that could be used for future animal studies in infertility.

**Methods:** Adult female Rattus norvegicus were used in this study. All were given VCD injections at 80 mg/kg intraperitoneally for 15 days. An examination of Follicle Stimulating Hormone (FSH) levels will be carried out on days 0, 3, 5, 7, and 15. To mimic IVF process, all rats got an injection of 10 IU of Pregnant Mare Serum Gonadotropin (PMSG) and 10 IU human Chorionic Gonadotropin (hCG) to confirm DOR. Ovarectomy was performed, dominant follicles were taken, denudation was carried out, and the oocytes were cultured for 12 hours. Then, oocytes were assessed through a microscope.

**Results:** Starting from day 5 post VCD induction, there was a significant increase in the FSH level in the group of rats that were induced with the administered VCD dose compared to the control group that experienced normal FSH fluctuations. The FSH concentration reached >50%. It was found that the number of degenerated oocytes had an average of 1.11. Oocytes that were in Germinal-Vesicle (GV) stage had an average of 1.89, while in the Metaphase I (M1) stage the average is 2.882. Lastly, the number of oocytes in the Metaphase (MII) stage which is fully matured had an
average of 1.117.

**Conclusions:** VCD can become an important tool for future studies that needs an animal model with DOR.

**Keywords**

VCD, DOR, Rats, Infertility
Introduction
Infertility remains a significant issue in the world of health, especially in the reproductive sector; currently, around 9–18% of couples worldwide experience infertility. Infertility requires comprehensive treatment to obtain optimal results. In cases of infertility, female factors were responsible for 46.7% of infertility cases, male factors were responsible for 19.0% of infertility cases, while the combination of male and female factors caused 18.2% of infertility cases, while 11.2% of cases of infertility remained unknown. Not all infertility problems can be solved using conventional methods, and currently, assisted reproductive technology (ART) is widely used to help couples with infertility. Although many new ART centers have been growing, there has been no significant increase in the success rate of the ART program in the last 40 years since ART began to be practiced. In general, worldwide, the success rate of ART programs only ranges from 30–40%. Some of the factors that have been known to influence the success rate of ART are the ovarian stimulation protocol, the quality, and maturity of the oocytes, the number of embryos produced, embryo transfer techniques, maintenance of the luteal phase, drug dosage, laboratory ability of embryology and helper’s expertise. Based on data released in Indonesia in vitro Fertilization Association in 2021, it is known that from all ART centers in Indonesia, the condition of diminished ovarian reserve or poor ovarian reserves accounted for around 4.5% of all indications of ART in infertile pairs in Indonesia.

The ovarian response to stimulation will affect the fertilization rate; the higher the number of mature oocytes with good quality obtained, the higher the chances of fertilization. The increasing number of fertilizations will increase the number of embryos produced, so the chances of pregnancy will increase.

Various efforts have been made to improve the process of folliculogenesis and steroidogenesis to produce oocytes that are mature and ready to be fertilized, including research conducted by Benninghoff et al. by providing calcium immunophore A23187 and ionomycin in the ovarian tissue of Atlantic croaker, which results in an increase in the accumulation of steroid hormone production up to 2 to 5 times. In addition, studies conducted on women diagnosed with polycystic ovarian syndrome (PCOS) with insulin resistance and receiving Metformin therapy for 24 months obtained significant improvements in the folliculogenesis process and progesterone levels that signaled the occurrence of ovulation. Much research regarding steroidogenesis in infertile women needs to be done for future treatment since 4.5% of In Vitro Fertilization (IVF) patients in Indonesia suffer from Diminished Ovarian Reserve. (DOR) which has a low success rate in conceiving. There are only a few studies that have been done regarding this matter that showed a significant and promising result. Human subjects themselves have not been used in research settings due to the ethical implications. Thus, the application of an animal study might aid the gap in experimenting.

To start research on infertility, it is difficult to find animal conditions that support research on infertility because it is difficult to find animals in an infertile state. Therefore, approaches and interventions are needed to obtain a model animal that can be used in research on infertility.

Vinylcyclohexene diepoxide (VCD), which is a metabolite of 4-vinyl cyclohexene (VCH) is often used in the industrial field, and is classified among carcinogens substances according to the International Agency for Research on Cancer (IARC) and Hazardous Substace Data Bank (HSDB). VCD, which are also found in commercially products, is a chemical intermediate, a reactive diluent for epoxy resins and diepoxides. Based on studies done by the National Toxicology Program (NTP) it has been reported that in addition to the carcinogenic effects which are reported by the IARC and HSDB, VCD also has an ovotoxic effects. The increasing of oxidative stress with lipid peroxidation and hydrogen peroxide formation and inhibiting the formation of antioxidants which are induced by VCD, causing ovarian degeneration, hence DOR. In studies related to the reproductive system, it has been reported that abnormally increase of oxidative stress induced by VCD and inflammation, inhibit follicle development and cause degeneration in the ovary, although a certain amount of Reactive Oxygen Species (ROS) is needed during follicle development and ovulation. It has also been reported that increased oxidative stress and inflammation in the ovary activate apoptotic pathways in follicular cells and thus ovary degeneration. This study looked at the use of VCD in inducing targeted DOR conditions in animals, especially rats, to create a model rat that could be used for future animal studies in infertile settings.

Methods
39 Adult female Rattus norvegicus rats aged 2–3 months with a body weight limited to between 200–250 grams were used in this study divided into two groups (experimental and control), resulting in 34 samples in intervention group. The total sample is calculated using the Federer formula. 5 rats were assigned as a control in a control group. There was no randomization done in this study as this study use purposive sampling. All female rats used were confirmed to have no problem in the folliculogenesis process by choosing rats that had experienced two estrus cycles for four days twice in a row. All rats that met the inclusion criteria were kept in cages based on the intervention group given. If the samples met at least one of the exclusion criteria, the sample was excluded from the study. The exclusion criteria were abnormal estrus cycle, died during the study process, unable to collect oocyte after the ovarium’s tissues were collected, and if the collected granulosa cell was contaminated.
Animals is a living thing with the ability to feel pain, and thus to ameliorate harm to animals, researchers are responsible for assessing the expected effects on laboratory animals. Researchers must minimize the risk of suffering and provide good welfare to animals. Suffering includes pain, hunger, thirst, malnutrition, temperatures too cold or too hot, fear, stress, injury, diseases, and restriction of the ability to behave normally/naturally. Researchers must consider not only the immediate suffering that may end during the experiment itself, but also the risk of suffering before and after the experiment, including trapping, labeling, anesthetizing, breeding, transporting, and stabilizing. This means that researchers must also consider the need for adaptation periods before and after the experiment. During the observation, animals are placed in special cages. Animals are given food as well as drink regularly. The well-being and health of the animal during the treatment and the observation period are strictly controlled.

Each rat from both intervention and control was give the standard food, drink, and ad libitum; all rat cages were kept for 12 hours with light and 12 hours without light. All rats in the control group and intervention group were given the same treatment, except for the intervention. No alteration was done among all rats in the control group. All rats in the intervention group that met the criteria were given 4-Vinylcyclohexene diepoxide (VCD) therapy, an ovotoxic substance. All rats were given VCD injections at 80 mg/kg intraperitoneally every day for 15 days. An examination of Follicle Stimulating Hormone (FSH) levels was carried out to assess the ovarian reserves of model rats and normal rats on days 0, 3, 5, 7, and 15.

All rats in the control group

On the seventh day after rats enter the diestrus phase, all rats got an injection of 10 IU of pregnant mare serum gonadotropin (PMSG) intra peritoneal to stimulate and synchronize the growth of follicles. Then, 48 hours after PMSG injection were given an injection of 10 IU of intraperitoneal human chorionic gonadotropin (hCG) to induce ovulation. This was done to confirm whether the modeled rat had reached the targeted DOR condition that represents the infertile human condition enough. The surgery was carried out to take ovarian tissue from rats and will be assessed histologically for its maturity.

The operation began with anesthesia in rats with a mixture of the anesthetic drugs ketamine (50–150 mg/kgBB) and xylazine (5–10 mg/kgBB) injected intraperitoneally. The ovaries were then identified, and an ovariectomy was performed.

After an ovariectomy, the dominant follicles were taken, denudation was carried out by using insulin syringe inside low temperature Laminar Air Flow (LAF) chamber. Then the oocytes were cultured for 12 hours by using Vitrolife G-1 Plus as a maturation medium in addition with Vitrolife Ovoil-Culture oil. After 12 hours, an assessment of the maturity of oocytes was carried out through a microscope examination. Then isolation from the granulosa tissue was carried out by puncturing from the follicles found, then from each mouse; granulosa cells were taken from the dominant follicle, which can be found under microscope examination.

Data analysis

A descriptive analysis was carried out to describe the characteristics of the subjects; a univariate data analysis was carried out to see a picture of the proportions of each variable to be presented descriptively. Data analysis were done using SPSS 26.0.

Ethical considerations

This research was conducted after obtaining approval and recommendations from the Animal Ethics Committee of the School of Veterinary Medicine and Biomedic Bogor Agricultural University on 6th of June 2022. This article is reported in line with the ARRIVE (Animal Research: Reporting of in vivo Experiments) guidelines.21

Results

The effectiveness of VCD that was administered on follicles to form DOR was shown.21 The total ovarian reserve of the rats were assessed using the level of FSH in their serum which was measured on day 0, 3, 5, 7, and 15. The results in Figure 1 showed that starting from day 5 post VCD induction, there was a significant increase in the FSH level in the group of rats that were induced with the administered VCD (red) dose compared to the control group (blue) that experienced normal FSH fluctuations. The FSH concentration reached >50%.

After inducing DOR with VCD, PMSG and hCG were administered and the number of oocytes was measured. Significant differences were observed in terms of follicle reserve before and after the hyperstimulation process. Comparison of oocytes obtained from ovaries after PMSG and hCG administration showed a significant difference. On the assessment of
each side of the ovary, the results in Table 1 showed that they were relatively the same. On the right side of the ovary, the average number of oocytes was 3.82 while on the left ovary the average number of oocytes was 3.12.

**Figure 2** showed oocytes that were successfully extracted and are still surrounded by granulosa cells. The oocytes were then cultured and denuded to separate the oocytes from the granulosa cells that still surround the oocytes.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (SD)</th>
<th>Range (Min–Max) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Oocytes</td>
<td>6.94 (2.16)</td>
<td>3–11</td>
</tr>
<tr>
<td>Right Ovarium</td>
<td>3.82 (1.3)</td>
<td>2–6</td>
</tr>
<tr>
<td>Left Ovarium</td>
<td>3.12 (1.62)</td>
<td>1–6</td>
</tr>
</tbody>
</table>

**Figure 1.** Follicle Stimulating Hormone (FSH) progression chart during Vinylcyclohexene Diepoxide (VCD) induction.

**Figure 2.** Extracted oocytes. The brightness and contrast of the figure were adjusted for the best view.
Figure 3. Oocytes that have been denuded and assessed for maturity. The brightness and contrast of the figure were adjusted for the best view.

Table 2. Oocyte maturation data.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (SD)</th>
<th>Range (Min–Max) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degeneration</td>
<td>1.11 ± 0.963</td>
<td>0–3</td>
</tr>
<tr>
<td>GV</td>
<td>1.89 ± 1.149</td>
<td>0–5</td>
</tr>
<tr>
<td>MI</td>
<td>2.882 ± 1.317</td>
<td>1–6</td>
</tr>
<tr>
<td>MII</td>
<td>1.117 ± 0.992</td>
<td>0–3</td>
</tr>
</tbody>
</table>

GV: Germinal-Vesicle stage; MI: Metaphase I; MII: Metaphase II.

After denudation of the oocytes that have been obtained, the evaluation of oocyte maturation was carried out. From the data in Table 2, it was found that the number of degenerated oocytes had an average of 1.11. Oocytes that were in GV stage had an average of 1.89, while in the MI stage the average is 2.882. Lastly, the number of oocytes in the MII stage which is fully matured had an average of 1.117.

Discussion

Infertility is a worldwide problem and 9–18% of couples are accustomed to it. Female factors account for 46.7% of the main cause of infertility. In Indonesia, 4.5% of females that underwent IVF cycles are females with DOR. Therefore, several studies are conducted to increase the chance of fertility in these women with DOR. However, due to the ethical limitation of the use of human as an experimental subject, and thus animal models to mimic a DOR setting in human is needed to conduct research on infertility.

The induction of DOR in model rats with VCD was proven in this research. VCD is a chemical used to produce tires, plastics, rubber, and insecticides. VCD is the only chemical that causes damage to the primary and primordial follicles in oocytes from rats. VCD is known to cause autotoxicity through inhalation, dermal administration, and oral intake in animals. VCD demonstrated this ovotoxicity effect in experimental rat animals. An indication that the model rat has reached DOR is the level of FSH that has increased >50% on the 15th day after VCD induction, while the rat that was not induced with VCD shows a physiologic fluctuation of the FSH concentration. VCD itself is known to induce DOR by stimulating oxidative stress and inhibiting the formation of antioxidants. They cause lipid peroxidation and the formation of hydrogen peroxide which causes inflammation and activates apoptosis in follicular cells.

FSH is the most used biomarker to assess ovarian reserve, especially day 3 FSH because it expresses the “basal” level or non-suppressed level of FSH. As the number of day 3 FSH increased, it indicated a diminished ovarian reserve.
Gonadotropin-releasing hormone (GnRH) stimulates the anterior pituitary to produce FSH and Luteinizing Hormone (LH) which targets the ovary in females. FSH itself is responsible for follicular development and recruitment and for stimulating the conversion of androgens to estrogen during folliculogenesis. When the level of estrogen rises, it inhibits the FSH and LH secretion due to the negative feedback to the hypothalamus and anterior pituitary. In women with DOR, there is a signal from the ovary to the brain to produce higher quantities of FSH because of the low number of follicles, therefore, a high FSH concentration indicated a low ovarian reserve.

After efforts were made to produce DOR model rats, hyperstimulation of the oocytes were done using PMSG and hCG to mimic an ART setting. This was done because DOR is a very significant problem in women who undergo IVF. Women with DOR tend to have fewer chances of becoming a parent. Exogenous gonadotropins are known to be used to stimulate ovulation in humans and animals which results in an increasing number of available oocytes. The mouse is an important animal model in fertility medicine for DOR conditions. The administration of PMSG combined with hCG is a well-known method to induce superovulation.

The ovarian response to PMSG and hCG affects the success rate of fertilization, and the number of mature oocytes with good quality is directly proportional to the chance of pregnancy. The result of hyperstimulation in the model rat of this research showed that the total oocytes induced had an average of 6.94. This result can be compared with several different studies. One study done by Popova et al. which studied the effect of PMSG as an inducer for superovulation on model rats, showed that the total number of ovulated oocytes produced using 15 IU PMSG and 20 IU hCG had an average of 40.7, which is more than 5 times the average of the total oocytes produced in our DOR rat models. Another study by Taketsuru et al. induced rats with 150 IU/kg PMSG and 75 IU/kg hCG and showed that the number of oocytes collected had an average of 24.2. This strongly suggests the negative impact of DOR on fertility as rats in our study showed that the average number of total oocytes is 6.94.

In our study, the maturity level of the oocyte was evaluated. There was an average of 1.12 oocytes that reached the MII stage. This means that < 20% of the total oocytes in hyperstimulated DOR rats are mature. In a study conducted by Zolbin et al. where the developmental stages of the follicles were evaluated after PMSG and hCG were given, the result showed that there was an average of five mature follicles, which is around 5 times more than the total number of mature follicles in our study.

**Conclusion**

In conclusion, VCD can induce DOR in a rat model as it is responsible for causing follicle loss. Therefore, VCD can become an important tool for future studied that needs an animal model with DOR.

**Data availability**

**Underlying data**

Open science framework: The use of Vinylcyclohexene Diepoxide to Create Diminish Ovarian Reserve Model on Rats, https://doi.org/10.17605/OSF.IO/548YK.

This project contains the following underlying data:

- Edited Extracted Oocyte after denudation.jpeg
- Edited Extracted Oocyte before denudation.jpeg
- FSH Progression Chart during VCD Induction.jpeg
- FSH Progression during VCD Induction.png.csv
- Oocyte Count Data.jpg.csv
- Oocyte Maturation Data.png.csv
- RAW Extracted Oocyte After Denudation.jpg
- RAW Extracted Oocyte before denudation.jpeg
Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

References

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