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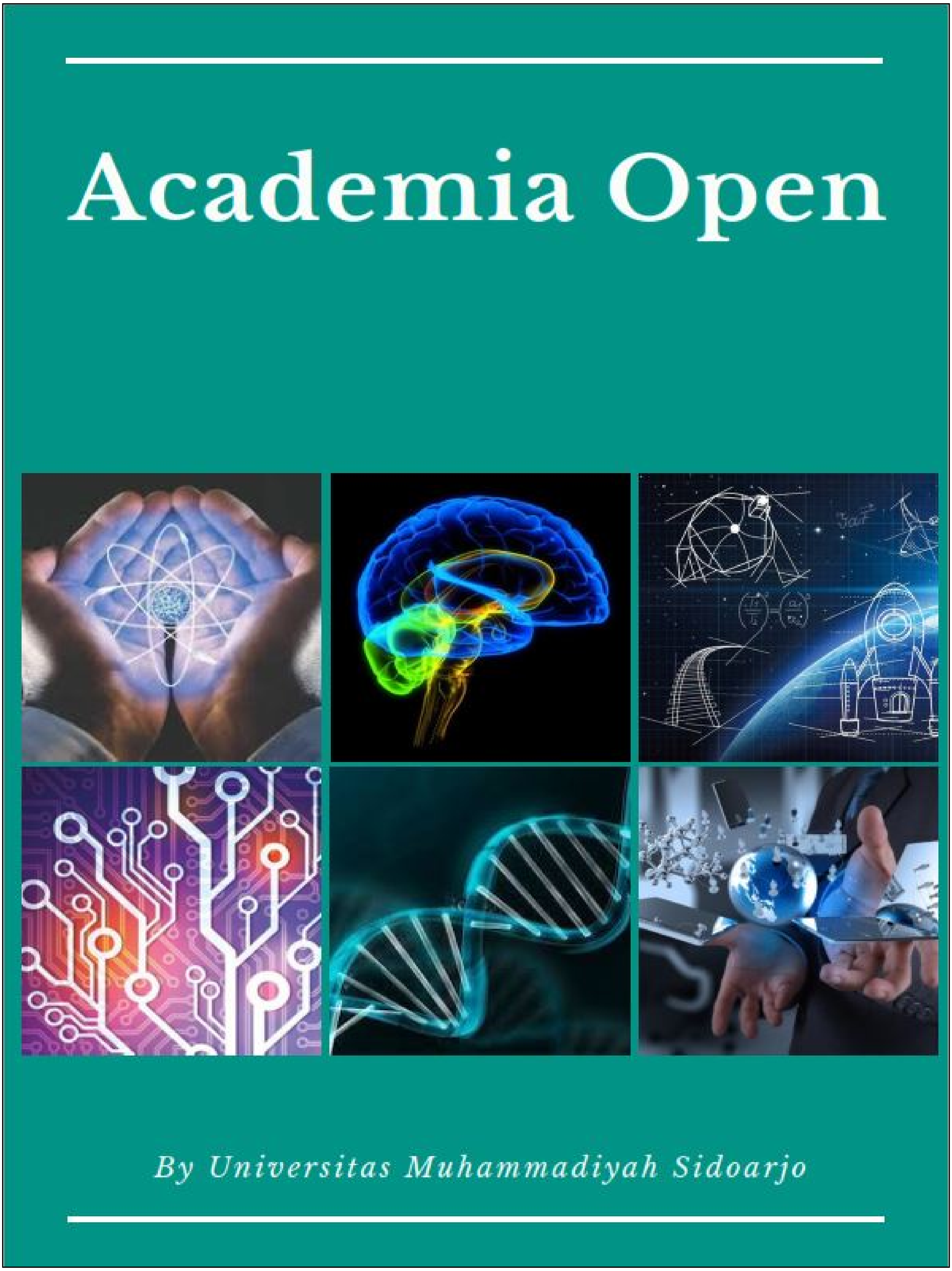

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Table Of Contents

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New Pigment (Khudhory Red Pigment) to Evaluate Oocytes Viability

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Abstract

General Background: Infertility remains a significant global health challenge, affecting approximately 60–80 million couples worldwide. Assisted Reproductive Technologies (ART) are widely employed to address infertility, and assessing oocyte viability is essential for ensuring the success of these techniques. **Specific Background:** The conventional trypan blue stain is commonly used for oocyte viability assessment, but concerns regarding its synthetic composition and potential cytotoxicity have prompted the search for safer alternatives. **Knowledge Gap:** Limited research has explored the use of natural pigments for oocyte viability testing, particularly those derived from antioxidant-rich sources like berries. **Aims:** This study aimed to develop and evaluate a novel natural pigment, named *Khudhory Red*, extracted from mulberry (Morus rubra), as an eco-friendly and safe alternative to trypan blue. **Results:** The pigment successfully differentiated viable and non-viable oocytes without causing cytotoxic effects, showing comparable performance to trypan blue in staining selectivity and clarity. **Novelty:** This is the first documented use of mulberry extract as a natural oocyte viability stain, demonstrating both safety and cost-effectiveness. **Implications:** The findings suggest *Khudhory Red* as a sustainable and biocompatible pigment for reproductive biology research, potentially replacing synthetic dyes in oocyte assessment.
Highlight :

- The study successfully prepared a new pigment (Khudhory Red) to evaluate oocyte viability.
- The new pigment showed no side effects on oocytes and acted similarly to trypan blue.
- Khudhory Red is natural, organic, safe, cheap, and easily available.

Keywords : Infertility, Oocyte, Khudhory Red Pigment, New Pigment, Trypan Blue

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Introduction

Infertility is the inability to create a pregnancy and give birth after 12 months of marriage (intercourse). Infertility is a global problem. Around the world, there are approximately 60-80 million couples affect from infertility [1]. The most important factors of infertility are disorders of the reproductive system, and reproductive system diseases [2], [3].

Assisted reproductive technology (ART) is the widely employed modality to alleviate and treatment of infertility. Every year, tens of thousands are born as a result of assisted reproductive technology(4). ART are used to overcome infertility, its procedures include those infertility therapy in which oocytes are handled in the laboratory.

To assess the oocytes viability and membrane integrity of the oocytes were used the vital dye staining (trypan blue). Trypan blue is a dye used to measure the viable (live) oocytes by tagging non-viable (dead) oocytes only. Trypan blue cannot pass through cell membrane of the viable oocytes and penetrate cytoplasm, but in the non-viable oocytes, its inter the membrane through porous and penetrate the cytoplasm and color it blue [4].

Some types of the berries, such as blueberry(vaccinium corymbosum), blackberry (Rubus caesius), black chokeberry (Aronia melanocarpa), cranberry (Vaccinium macrocarpon), and mulberry (Morus rubra L.). Berries is a valuable medicinal plant that is widely plants in world, Mulberry fruit, contain a wide uses in medical filed, mulberry extract is considered one of the most important antioxidant. Some studies have shown that mulberry can increase the expression of the anti-apoptotic factor and reduce the expression of the pro-apoptotic factors [5], [6].

Extract of the mulberry fruits have hypoglycemic activity in in vitro and in vivo experiments, also its prevents diabetes by regulation of blood glucose through upregulation of antioxidative activity. The insoluble-bound of the berries are important natural sources of antioxidants for preventing diseases associated with oxidative stress . Mulberry extract have good hypoglycemic activity because it is rich in flavonoids and polysaccharides. In addition, it is can prevention and treatment diabetes mellitus. Also has effects on diabetic complications of the central nervous system by upregulating the antioxidative activity [7], [8].

In this study, work to finding a new pigment as an alternative to trypan blue, it is an organic, natural and more safety, approximately without side effects. In this work was used the mulberry extract as a pigment to evaluate the viability of oocyte because its natural and organic without side effects on the oocytes. Also it is available and cheap [9].

Berries

Bioactive compounds possess high antioxidants (the substances that can scavenge free-radicals) capacity. Some antioxidants help to decrease the incidence of oxidative stress induced damage. The berries are full of bioactive compounds that are belong to antioxidants such as fruit colorants (carotenoids and anthocyanins), and phenolic compounds [10]. The phenolic acid in the berries such as hydroxycinnamic acid and hydroxybenzoic conjugates. Flavonoids, such as Anthocyanins, flavanols, and flavonols. Other components are tannins (hydrolyzable tannine, and condensed tannins) [11], [12]. Also, there are antioxidants such as vitamins (ascorbic acid) and minerals with antioxidant properties, that are considered included bioactive compounds .

One of the most important components of berries is anthocyanin, which is a very strong antioxidant and is used to treat many diseases and is responsible for pigment and color gradations in berries. It is considered the active substance in pigmentation [13].

The chemical composition of berries. The berries contain low in calories, but high in sugars. Also, has a high content of fiber such as pectin, cellulose, hemicellulose, but small amounts of fat; some vitamins such as folic acid and ascorbic acid; organic acids, such as malic acid, tartaric acid, citric acid, and oxalic acid; trace amount of certain minerals. Figure (1) mulberry.

Figure 1. Mulberry

Materials and Methods

A. Oocytes collection

From a local abattoir collect the ovaries and transplantation to the laboratory during 45 minutes by container containing a normal saline (NS), with $37^{o}C$. separation the ovaries follicles from ovaries, using a 5 mL syringe with a 23-gauge needle. Then, the oocytes washed three times with simple medium assisted reproductive technologies (SMART medium) . this work were used the cumulus-oocytes complexes (COCs) with homogenous cytoplasm.

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Figure 2. Sheep ovaries after being collected and washed.

B. Aspiration of Oocytes

Using a sterile disposable 5 mL syringe with a 23 gauge hypodermic needle for aspirating the oocytes with follicular fluid. By aspiration technique (figure 3), Aspirated oocytes from all the visible follicles on the ovarian surface with (4-6 mm) in diameter. This syringe contains (5%) BSA with 0.5 mL SMART medium and (20 IU/ml) heparin. Empty the contents of each syringe in a petri dish, examine this content for collected oocytes was under the dissected microscope using a modified pasture pipette or automatic micropipette then washed the oocytes (3-4) times with medium (figure 4).

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Figure 3. Aspiration of the oocytes from the ovary.

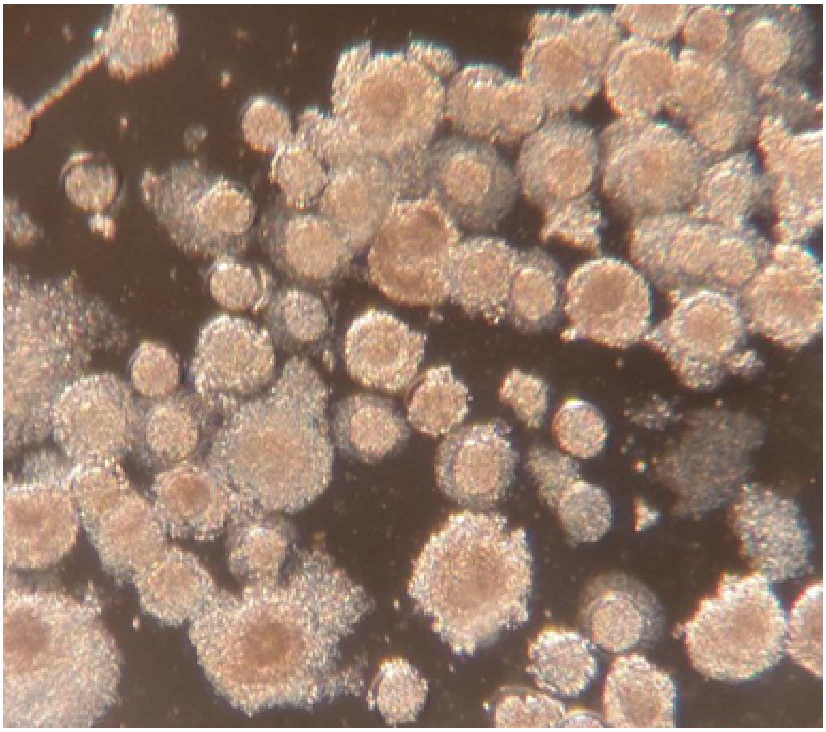

Figure 4. Retrieved oocytes under a dissected microscope(X=100)

C. Preparation of trypan blue

Use the trypan blue stain to differentiate the non-viable oocytes from viable oocytes before using the new stain (khudhory

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red) for evaluation purpose. The trypan blue stain is prepared through adding (0.2 g) of powder of the trypan blue to the normal saline (50 ml). then, for filtration used the filter paper. After this, placed inside a refrigerator in a dark bottle.

D. Preparation of khudhory red (new stain)

This study was used mulberry. The mulberry were collected from any markets. Then, it is washed twice or three with tap water inside the laboratory, after that, it is squeezed manually in a small dish and then the juice is taken, put it in a tube, and leave for two hours. In the last, withdrawn the supernatants, then filtration by filter paper, and store (without any addition) in the refrigerator in a dark bottle, figure (5).

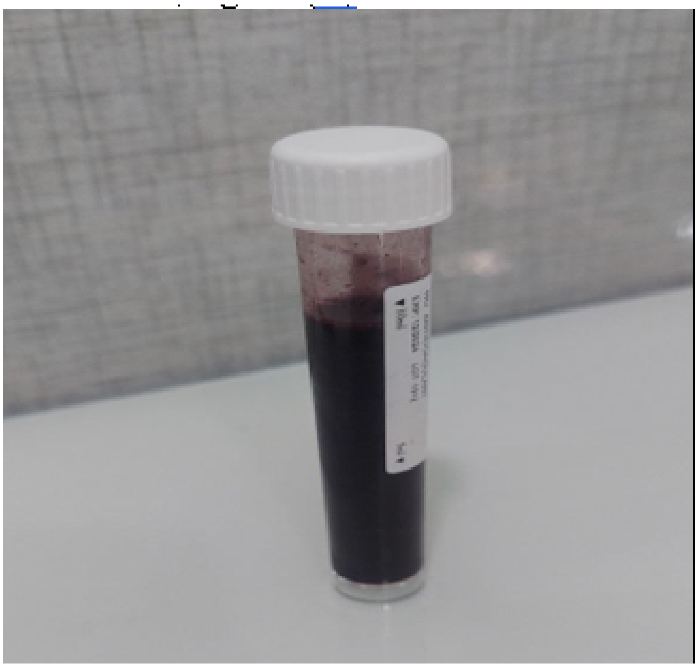

Figure 5. New Pigment (khudhory red pigment)

E. Viability test

In the beginning, examine the viability for all oocytes with trypan blue to classified it into live (viable) oocytes (unstained) (figure 6), and dead (non-viable) oocytes (full stain) are removed. Second step, using the khudhory red to evaluate viability of oocytes. Then, these oocytes (viable) are left to die, by leaving them in unsuitable conditions such as low\ high temperatures or medium without nutrients outside the incubator. third step, again stain with khudhory red, unstained oocytes are viable (figure 7), and full stain oocytes (red in colour) are non-viable (dead)(figure8).

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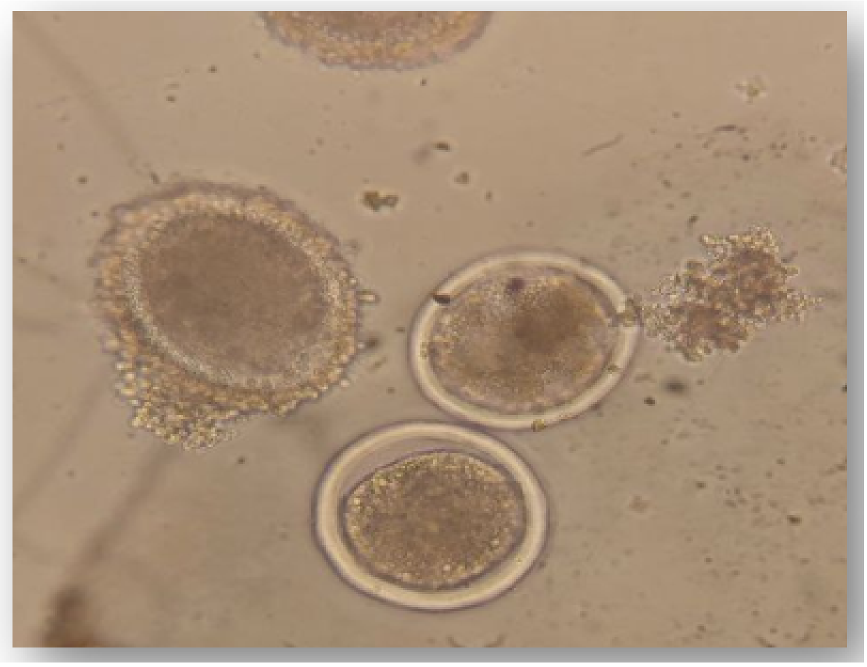

Figure 6. Oocytes without any stain

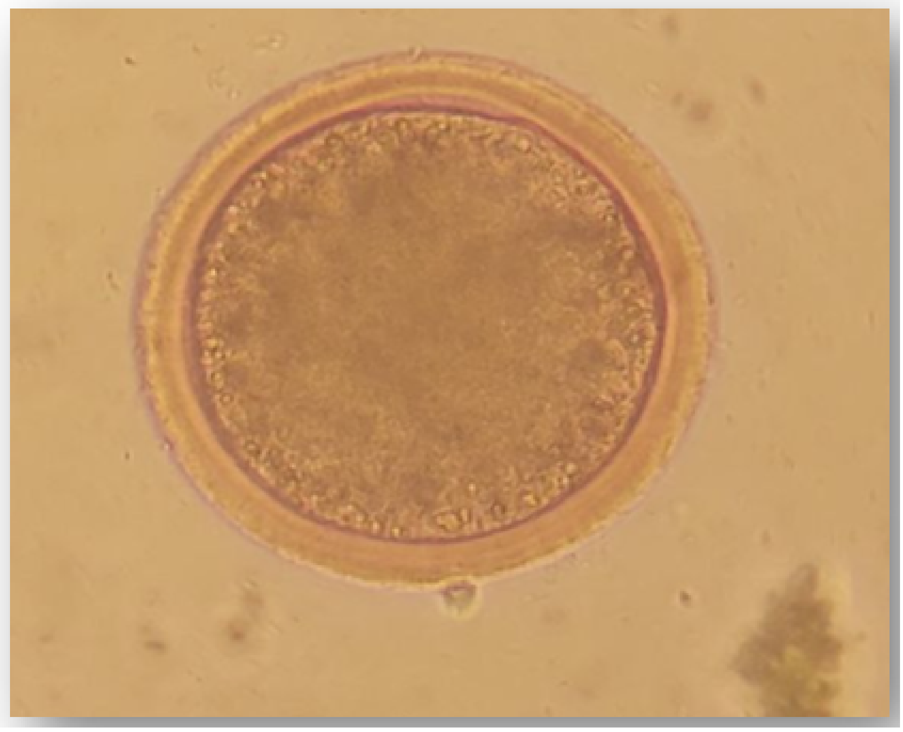

Figure 7. Viable oocyte after khudhory red

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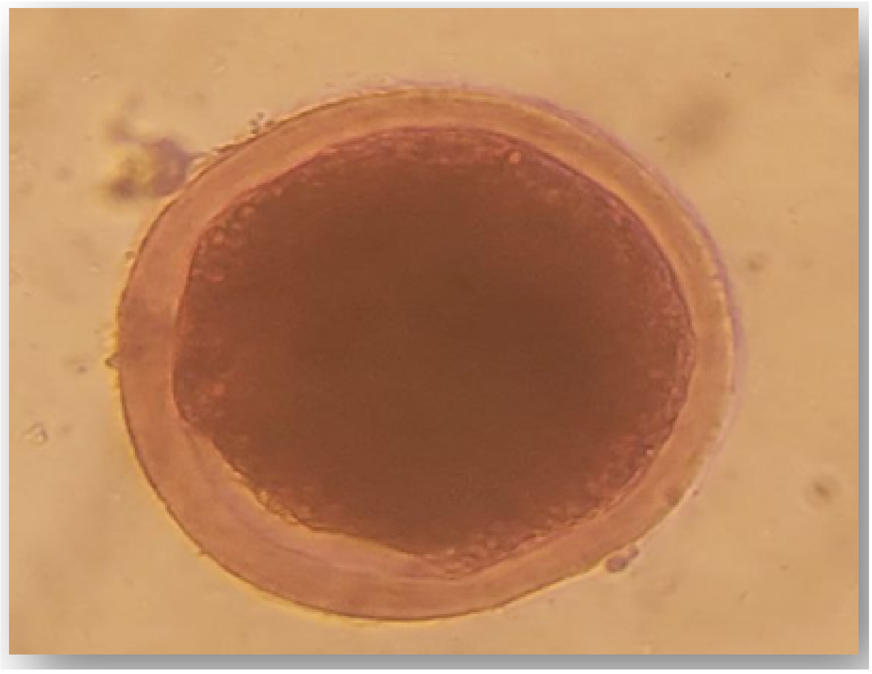

Figure 8. Non-viable oocyte after khudhory re

Results and Discussion

During this study, aspirated (82) oocytes from (44) ovaries, were obtained from a local slaughterhouse. The highest percentage of recovered oocytes was (250 %), and the lowest percentage of recovered oocytes was (112.5 %) table1). The highest and lowest percentage of recovered mature oocytes was(44.44 %), and (10 %) respectively [14]. While the highest and lowest percentage of recovered immature oocytes was (76.92 %), and (44.44 %) respectively. (11.11 %) is the highest percentage of recovered atretic oocytes, and (0 %) is the lowest percentage of recovered atretic oocytes, table (1)

[Table 1 : is here]

The total number of oocytes was (82). number of mature oocytes was (20) from the total number, while the number of immature oocytes was (57), and number atretic oocytes was (5), obtained from (44) ovaries. Most of the recovered oocyte was immature and lowest number oocytes was mature with few atretic oocytes [15], [16].

Classification of oocytes

Classification recovered oocyte was after aspiration into; oocytes with cumulus cells (-CC), and oocytes without cumulus cells (+CC). In this work, the percentage of oocytes with cumulus cells was (81.99 %), while the percentage of oocytes without cumulus cells was (18.01 %), figure (9).

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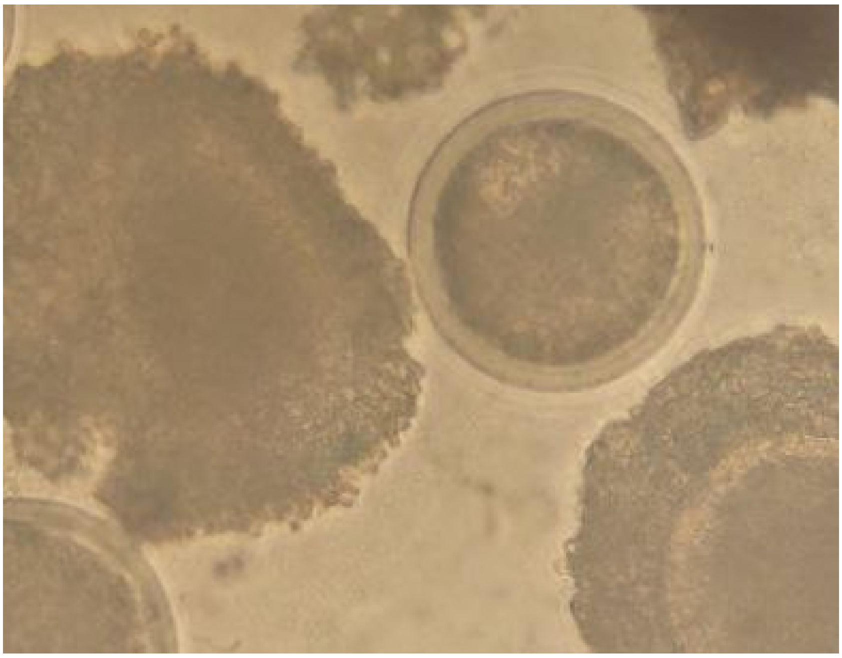

Figure 9. oocytes with and without cumulus cells (+CC, -CC)

Viability of oocytes :

After were collection of oocytes, examine the viability for all oocytes after it has been prepared and washed in laboratory, with trypan blue to classified it into live (viable) oocytes (unstained)(figure 10 A), and dead (non-viable) oocytes (full stain)(figure 10B). To assess the oocytes viability and membrane integrity of the oocytes were used the trypan blue stain (vital dye). Trypan blue is a dye used to measure the viable (live) oocytes by tagging non-viable (dead) oocytes only(6).

Trypan blue cannot pass through cell membrane of the viable oocytes and penetrate cytoplasm, but in the non-viable oocytes, its inter the membrane through porous and penetrate the cytoplasm and color it blue. As a result, all blue oocytes were dead, while non-blue oocytes are viable [17].

Second step, remove all non-viable oocytes, and then using the khudhory red to evaluate viability of oocytes. Then, these oocytes (viable) are left to die, by leaving them in unsuitable conditions such as low\ high temperatures or medium without nutrients outside the incubator [18], [19] [20]. Third step, again stain with khudhory red, unstained oocytes, the stain cannot inter cytoplasm are viable (live)(figure 11A), and full stain oocytes, the khudhury red pass through membrane by porous and penetrate cytoplasm (red in colour) are non-viable (dead)(figure 11B) [21], [22], [23].

Thus, it is said that every oocyte that acquires a red color is dead (non-viable), and any oocyte that does not acquire a red color is a live (vible).

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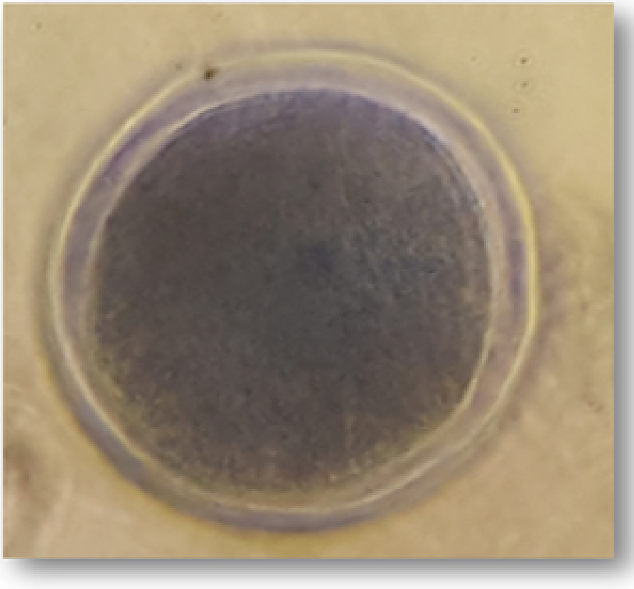
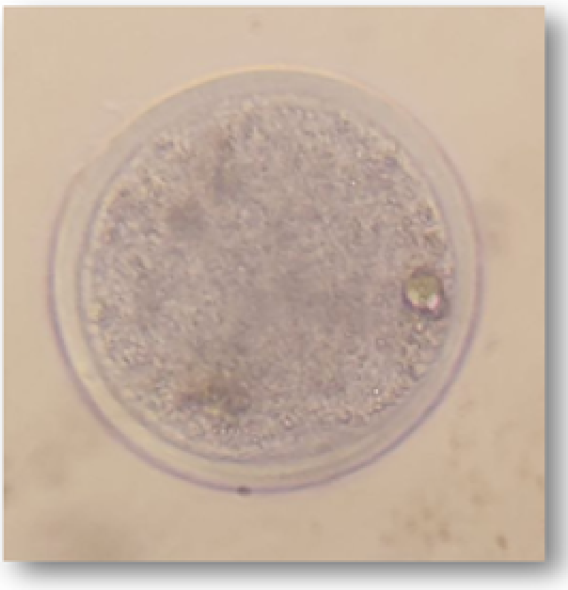


A

Figure 10. Non-Viable Oocyte (dead) After Trypan Blue



B

Figure 11. Viable Oocyte (live) After Trypan Blue

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Vol. 10 No. 2 (2025): December
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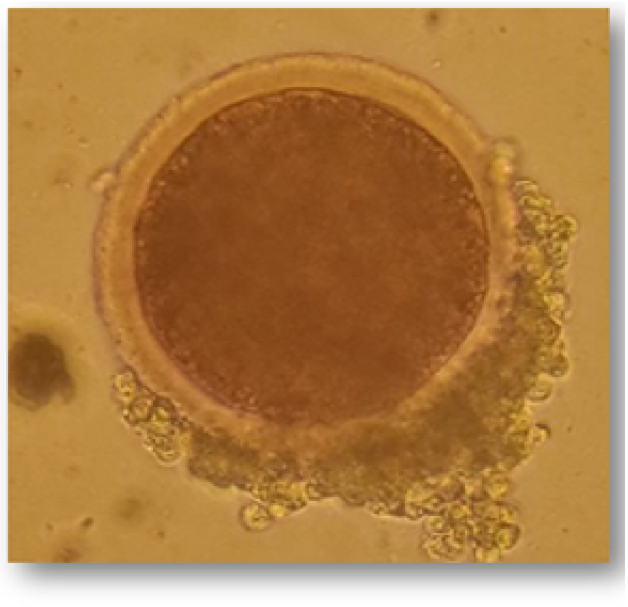
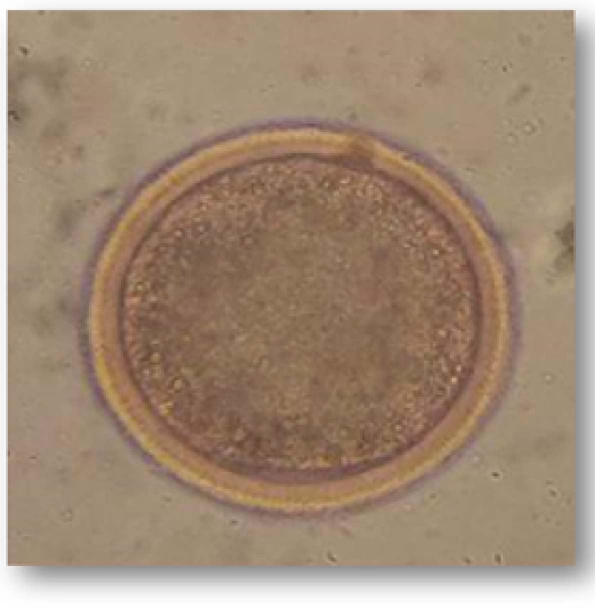


A

Figure 12. Non-Viable Oocyte (dead) After Khudhory Red



B

Figure 13. Viable Oocyte (live) After Khudhory Red

Conclusion

1. From the results of the present study it was concluded that the new pigment (khudhory red) has been evaluated the oocytes viability and no side effects on oocyte viability.

2. Successful preparation of new pigment.

3. Successful evaluation of oocytes viability by khudhory red pigment.

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4. new pigment (khudhory red) act as trypan blue.

Abbreviations

1. ART Assisted reproductive technologies

2. NS Normal Saline

3. SMART Simple Medium Assisted Reproductive Technologies

4. COCs Cumulus-Oocytes Complexes

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