QUALITATIVE EVALUATION OF SLAUGHTERHOUSE BOVINE OVARIES BY HISTOLOGICAL STUDY

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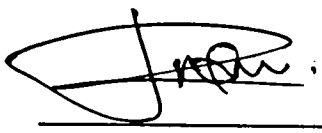

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ABSTRACT

This study was conducted at the Anatomy and Physiology Laboratory, Faculty of Sustainable Agriculture, Universiti Malaysia Sabah Sandakan Campus from August 2016 until October 2016 to do a reach work on the analysis of the slaughterhouse bovine ovaries in term of the quality by histochemistry studies. The objectives that been set for this study were to identify the quality of follicles in bovine ovaries and to find out the relationship between the quality of follicles and ovarian state. The ovaries were collected from slaughterhouse and morphological evaluations such as weight and number of visible follicles on the surface of the ovaries were counted. The ovaries been classified into two groups as ovary with corpus luteum (CL+) and without corpus luteum (CL-). The recorded percentages of ovaries that been retrieved from the slaughterhouse is 75.0% CL- and 25.0% CL+. The visible follicles on the surface of the ovaries that been recorded at average level for CL+ (23.0 ± 2.4) is lower than the CL- (26.0 ± 2.2). From the collected ovaries represented several ovaries from both groups been used for histochemistry study. The ovaries then been prepared for histological study by fixation, dehydration, clearing, embedding, and blocking with paraffin wax. The waxed sample block been trimmed with microtome at 6μm and stained with Hematoxylene and Eosin (HE). The sample observed under microscope and the number of follicles and the granulosa cells integrity been recorded. From the finding, the CL- ovaries were contained good quality follicles (8.8 ± 0.3) at grade ≥3 granulosa cells intergrity, while CL+ ovaries recorded (4.0 ± 0.8) at grade ≥3 granulosa cells integrity. The microscopic counted follicles in CL- ovaries (198 ± 5.8) found higher than that of CL+ ovaries (170 ± 7.8). From the experiment, it is found that the CL- group ovaries contained higher number of good quality follicles than that of CL+ group ovaries. This finding further suggests that, the slaughterhouse ovaries without CL might be suitable to obtaining the quality cumulus-oocyte-complexes (COCs) for *in vitro* embryo production (IVEP) experiments.

ABSTRAK

Kajian ini telah dijalankan di Makmal Anatomi dan Fisiologi, Fakulti Pertanian Lestari, Kampus Sandakan Universiti Malaysia Sabah, dari Ogos 2016 hingga Oktober 2016 untuk menganalisis ovari lembu sembelihan dari segi kualiti melalui kajian histochemistry. Objektif yang telah ditetapkan dalam kajian ini adalah untuk mengenal pasti kualiti folikel dalam ovari lembu dan untuk mengetahui hubungan diantara kualiti folikel dan keadaan ovari. Ovari telah dikumpul dari rumah sembelih dan telah dilakukan penilaian morfologi terdahulu seperti mengambil berat ovari dan kiraan folikel yang boleh dilihat pada permukaan ovari. Ovari yang dikumpul telah diklasifikasikan kepada dua kumpulan seperti ovari dengan korpus luteum (KL +) dan tanpa korpus luteum (KL-). Peratusan ovari yang telah diambil dari rumah sembelih adalah 75.0% KL- dan 25.0% KL +. Folikel yang boleh dilihat pada permukaan ovari telah direkodkan dengan purata KL + (23.0 ± 2.4.0) adalah lebih rendah daripada KL- (26 ± 2.2). Dari ovari yang dikumpul beberapa ovari telah dipilih daripada kedua-dua kumpulan dan digunakan untuk kajian histokimia. Ovari telah disediakan untuk kajian histologi dengan pengawetan, dehidrasi, penjelasan, penerapan, dan menyekat dengan lilin parafin. Blok sampel yang telah dililin paraffin dipotong dengan mengunakan mikrotom pada 6µm dan diwarnakan dengan Hematoxylene dan Eosin (HE). Sampel dikaji di bawah mikroskop dan bilangan folikel dan gred sel-sel granulosa direkodkan. Dari kajian ini, ovari KL- dinyatakan kualiti folikel baik (8.8 ± 0.3) pada gred sel granulosa ≥3, manakala ovari KL+ mencatat (4.0 ± 0.8) pada integriti sel-sel granulosa pada gred ≥3. Folikel yang dikira melalui mikroskop bagi oavri KL- (198 ± 5.8) adalah lebih tinggi daripada ovari KL + (170 ± 7.8). Dari eksperimen ini, didapati bahawa ovari kumpulan KL- menunjukkan bilangan folikel berkualiti dari segi nombor adalah lebih tinggi daripada ovari kumpulan KL+. Ini membuktikan bahawa, ovari tanpa KL dari rumah sembelihan sesuai untuk mandapatkan kumulus-osit-kompleks (KOKs) bagi tujuan menjalankan experiment pengeluaran *in vitro* embrio (IVEP).

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%	Percent
<	Less Than
≤	Equal and Less Than
±	Plus or Minus
≥	Equal and More Than
°	Degree Angle
°C	Degree Celsius
µg	Microgram
µm	Micrometre
CL	Corpus luteum
CL-	Corpus luteum absent
CL+	Corpus luteum present
COCs	Cumulus Oocyte Complexes
DVS	Department of Veterinary Services Malaysia
FSH	Follicle Stimulating Hormone
g	Gram
GnRH	Gonadothrophine Releasing Hormone
h	Hours
H&E	Hematoxyline and Eosin
Ha	Alternative Hypothesis
Ho	Null Hypothesis
IU	Insulin Units
IVEP	*In-vitro* Embryo Production
LH	Luteinizing Hormone
ml	Millilitre
mm	Millimetre
MOET	Multiple Ovulation Embryo Technology
OPU	Ovum Pick-up Units
P	Probability
SE	Standard Error
vs	Versus

CHAPTER 1

INTRODUCTION

1.1 Current Status of Beef and Dairy Industry in Malaysia

Malaysia is a developing country that focuses on the development of agriculture sector as well as livestock production. In Malaysian livestock industry, the major focus is being given on ruminant production since mostly non-ruminant livestock production is performing well. Beef is an important protein food source for humans, and an important commodity in Malaysia. Meanwhile, the consumption and use of dairy products within the Malaysian is also increasing gradually. In Malaysia, it was expected that local production of beef can only fulfil 30% of the country's total beef demand for 2012 (Jamaludin *et al.*, 2014).

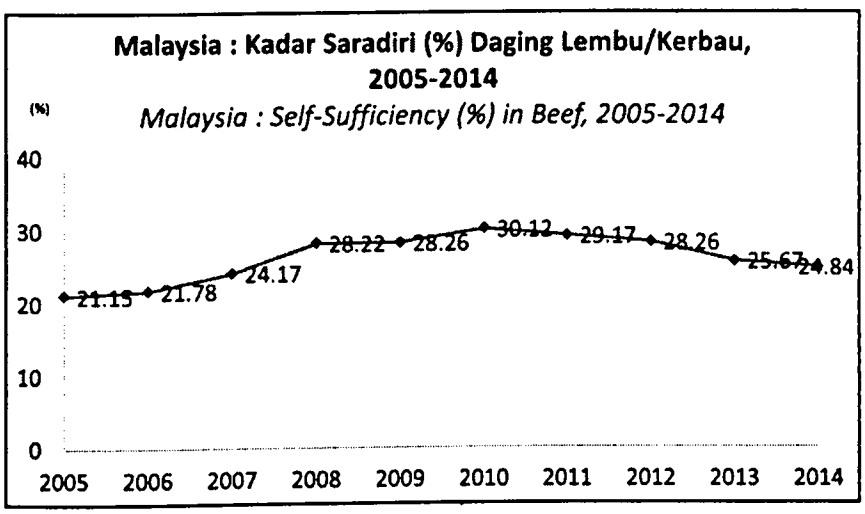

Figure 1.1 (a): Malaysia : Self-Sufficiency in Beef (%), 2005-2014 *Source: Department of Veterinary Services of Malaysia, 2014*

Based on the information given by the Department of Veterinary Services of laysia (DVS) on the 2014, shows that, the self-sufficiency level of Malaysia on the beef is nearly 24.84% while the higher self-sufficiency level was recorded on the year 2010 around 30.12% which can be found from the Figure 1.1 (a).

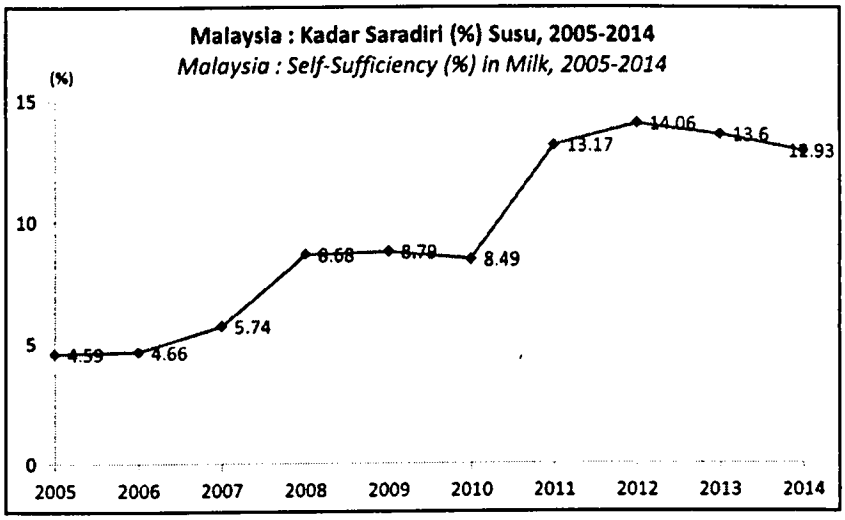

Figure 1.1(b): Malaysia : Self-Sufficiency in Milk (%), 2005-2014 *Source: Department of Veterinary Services of Malaysia, 2014*

DVS Malaysia also recorded the self-sufficiency of Malaysia on dairy (milk) production on 2014, shows that the highest self-sufficiency level was recorded on the year 2012 around 14.06%, while on the year 2014 it was just 12.93% which been stated on Figure 1.1 (b). The consumption rate of the livestock products are increasing yearly in Malaysia which due to higher demand (Ariff *et al.,* 2015).

The major factor that influence on the rise in demand and low in self-sufficiency is due to the rapid population growth and increase in consumption rate of livestock products in Malaysia. The lower production of livestock animals is also another factor that leads to this low self-sufficiency level in both beef and dairy production. The low production of livestock animals is pointing that, the Malaysian livestock animals are performing low in reproduction and production (Jamaludin *et al.*, 2014 and Ariff*et al.,* 2015). One of the solutions to increase the livestock production is increasing the number of stocks. This is where we should implement new biotechnology ideas in improving the reproduction performance of currently available livestock animals which we focusing on beef and dairy cattle.

Pass few decades, Malaysian Agricultural Research and Development Institution, MARDI trying to improve the locally available breed of cow with plenty of animal biotechnology techniques. Due to a lot of restriction and failures they could not fulfil their goals. MARDI has done experiment on oestrus synchronization and conception rate, the ovarian follicular wave patterns, oestrus interval, and ovulation rate in Kedah-Kelantan cows and MARDI has developed Brakmas cattle (Nor Amna A'liah M. N. and Mohamad Hafizan R. 2015). Even plenty of latest biotechnology tools

being use in animal reproduction and biotechnology works, the common technologies are multiple ovulation and embryo transfer (MOET), ovum pick-up units (OPU), *in vitro* embryo production (IVEP), cloning, transgenesis and also artificial insemination (Sirad and Blondin, 1996). There are few restrictions in those technologies application because of the inducing hormones artificially to speed up the ovulation process in order to retrieve more and more embryos and oocytes. The cows that been used in those technologies, mostly the oocyte or embryo donor cow will face the difficulty to perform well to the hormones for the long term. This is because of continues use of the hormones artificially, will affect their reproduction system. Alternative ways should be found to improve the technology.

1.2 Problem Statement

In Malaysia, to do *in vitro embryo* production (IVEP), we need continues supply of oocytes and sperms, in order to increase the beef and dairy production. It is easy to get sperms in large amount due to the latest technology we can dilute the semen that contains 0.5 million to 300 million sperm cells per ejaculation on in average, but the oocyte is only one will be ovulated at time of per ovulation cycle. Besides that, the resistance of donor cows in producing large amount of oocytes in continues use of MOET technique. Meanwhile OPU which is considered the best option to produce IVEP embryos, but it need to be studied and establish the proper protocols and techniques. To improve the protocol and techniques, it may useful to use the slaughterhouse bovine ovaries. The major problem in doing IVEP from slaughterhouse ovaries is clearly noticed the scarcity of quality oocytes. Therefore, there is suggestion of using slaughterhouse bovine ovaries to retrieve oocytes been proposed by several scientists world-wide (Hufana-Duran *et al.,* 2005, Khandoker *et al.,* 2011, and Khandoker *et al.,* 2012).

Besides that, the use of slaughterhouse ovaries in animal reproduction biotechnology studies is collecting cumulus-oocyte-complexes (COCs) (Mondal *et al.*, 2008). Collecting the COCs in conducting IVEP embryos is for the *in vitro* maturation, *in vitro* fertilization, and *in vitro* culture of the oocytes. This is suggesting that, the potentiality of the slaughterhouse ovaries on the retrieval of COCs. Previously few experiments on collection and evaluation of cumulus-oocyte-complexes (COCs) from slaughterhouse goat ovaries conducted and reported that the higher number of follicles

was found in the ovaries without corpus luteum (Islam *et al.*, 2007 and Nur Farah Atiqah Y. 2016). The findings show that, the number of follicles, quality of COCs and oocytes are higher in ovaries without corpus luteum. These results show that, the ovaries from the slaughterhouse is potentially can be used in IVEP research works. The unknown sources of the ovaries from the slaughterhouse, questioning the scientist of study more about the cellular level potential of those ovaries. Therefore, to study and prove the previous findings, histological study is being conducted on the slaughterhouse ovaries. This will be helpful in using the slaughterhouse ovaries in making cell line and culturing studies to improve the IVEP embryos. Besides that, the good genetic potential of the studied oocytes can be used in gene transferring to produce transgenic animals.

1.3 Justification

The lower production of beef and dairy products in locally is increasing the demands for meat and milk in Malaysia. In the state of Sabah, Malaysia few well-known dairy companies such as Desa Dairy Cattle, Keningau Integrated Livestock Centre (KILC), and Evergreen Livestock Sdn., Bhd are producing large amount of milk to face the demands on the dairy products locally. Meanwhile the Sawit Kinabalu oil palm plantation company actively involves in producing beef cattle to face the beef demand in Sabah, Malaysia. Even though they are trying to improve the production, the lack of biotechnology tools in their farming system, it suppresses the production. Therefore the collaboration of Faculty of Sustainable Agriculture of Universiti Malaysia Sabah should be used as the initial step in introducing the IVEP technology using slaughterhouse bovine ovaries.

Before the implement of the technology the further studies on the potentiality at stated earlier should be conducted. Studying on the slaughterhouse bovine ovaries with histologically will have a practical and a scientific relevance. Although the morphological analysis of the ovary been used to determine or discriminate the developmental potential of the oocyte and the relationship between the condition or type of the ovary, but yet do not have correspondence between the visual criteria and the oocyte condition of an ovary. Therefore the histochemical study will be an initial work on predict the development and state of ovary on the oocyte quality. This study also being continue from the senior colleague Nur Farah Atiqah Y. (2016) who studied

the effect of ovarian type on the number and quality of cumulous-oocyte-complexes (COCs) and *in vitro* maturation of bovine COCs showed that the ovaries without corpus luteum suitable to be used for IVEP experiment. In addition, number of oocytes recovered per ovary having CL and without CL in buffaloes, and their relevant percentage of different grades of oocytes recovered from abattoir-collected ovaries are having different number was stated by Sahoo, and K. Singla (2013) from India. In order to prove the findings, histochemical study is being conducted on slaughterhouse bovine ovaries to analyse the quality.

1.4 Objectives

Considering the above facts and circumstances the present study was undertaken with the following objectives:

1. To identify the quality of follicles in bovine ovaries.
2. To find out the relationship between the quality of follicles and ovarian state.

1.5 Hypothesis

$H_{o\,(i)}$: There is no difference on the ovarian type and the quality of the follicles.
$H_{a\,(i)}$: There is difference on the ovarian type and the quality of the follicles.

$H_{o\,(ii)}$: There is no difference between the layers of granulosa cells and the quality of the follicles.
$H_{a\,(ii)}$: There is difference between the layers of granulosa cells and the quality of the follicles

CHAPTER 2

LITERATURE REVIEW

Detailed anatomical, histological studies of the ovaries and the factors of ovaries that effect the ovulation and reproduction of cattle had been done previously by other researchers. Several research studies been done and reported on the histological studies on animal reproductive organs and related works on reproduction that so far been studied are reported accordingly.

2.1 Reproductive System

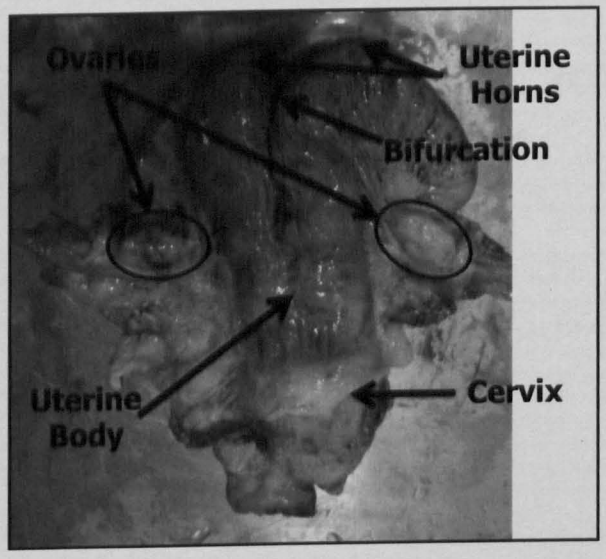

Figure 2.1 Female (Cow) reproduction organ

Source: Personal Collection

In reproduction of animal females plays vital role in producing the oocyte and gestating the young offspring until the calving. Therefore, female reproduction should be studied deeply in producing good livestock breeds. The most important reproduction organ of

female is ovary. This been stated by Krishna. *et al.* (2015). Figure 2.1 shows the reproduction organ of cow.

2.1.1 Ovary (Ovaries)

Krishna *et al.* (2015) from Department of Zoology, Banaras Hindu University, stated in their article publication on *Reproduction: Mammalian Ovary*, that the ovary is a multi-compartmental female gonad with broad range of distinct biological properties. The primary function of the female gonad is the differentiation and release of the mature oocyte or egg during each reproductive cycle that is fully competent for fertilization and successful propagation of the species. In addition, the ovary produces steroids that allow the development of female secondary sexual characteristics and support pregnancy. Besides that, in the ovary, each germ cell is in contact with multiple supporting cells, known as granulosa cells and thecal cells, forming an ovarian follicle. There are two main functional units within the ovary that known as follicle and the corpus luteum. Figure 2.1.1 shows the photographic image of bovine ovaries with visible follicles.

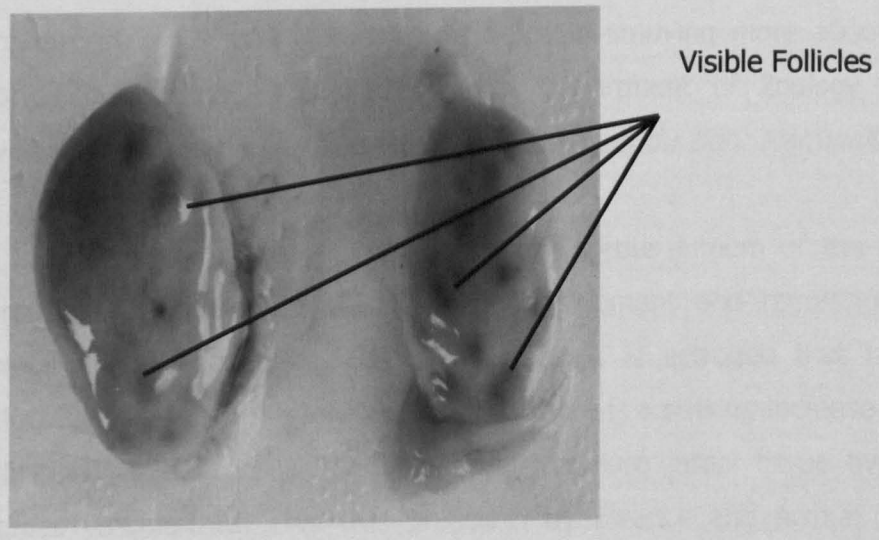

Figure 2.1.1 Photographic image of bovine ovaries with visible follicles
Source: Personal Collection

Reece (2009) stated that the ovaries are paired organs situated on either side of the uterus. They are attached on one edge, the hilus, to the broad ligament of the uterus by a fold of peritoneum, the mesovarium. The inner medulla is highly vascular and composed of a loose connective tissue core. Examine the outer cortex of the ovary

which is composed of stroma and numerous follicles in various stages of development. The cortex also contains many oocytes embedded in this cortical stroma.

2.1.2 Corpus luteum

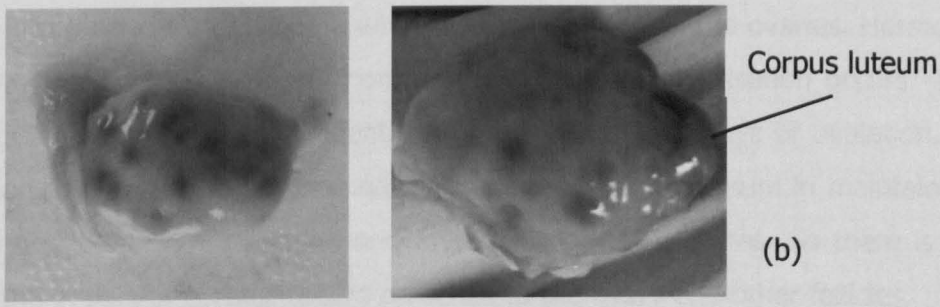

Figure 2.1.2 a. Ovary without corpus luteum and b. Ovary with corpus luteum
Source: Personal Collection

Figure 2.1.2 shows an ovary with and without corpus luteum on its surface. The corpus luteum is formed from the mature follicle, after it has ovulated; one of its main functions is to secrete hormone, progesterone, which is essential for preparation of uterus for the initial stages of pregnancy in a fertile cycle. Besides that, the functions of corpus luteum stated in this article helps in studying more about CL. This been reported by Krishna *et al.* (2015) from Department of Zoology, Banaras Hindu University, stated in their article publication on *Reproduction: Mammalian Ovary.*

Ultrastructural study of the transitory corpus luteum of the menstrual cycle affords an opportunity to examine the development and regression of organelles involved in steroidogenesis. Before ovulation it is estrogen that is predominantly produced in the follicle wall. After ovulation there is a striking increase in progesterone production by the corpus luteum as the granulosa luteal tissue hypertrophies and becomes vascularized. This was proposed by Eleanor and Arthur (1969), of their publication on The Journal of Cell Biology pages from 696-715. They also studied the ovary under ultra-imaging technology to understand the ovarian state with presence of corpus luteum. They focused on the hormonal impact on ovaries rather than the follicular state of the examined ovaries.

Lutz (2009) also stated that, the corpus luteum is formed by both granulosa cells and thecal cells after ovulation has occurred. The wall of the follicle collapses into

a folded structure, which is characteristic for the corpus luteum. Theca interna cells and granulosa cells triple in size and start accumulating lutein within a few hours after ovulation. They are now called granulosa lutein cells and theca lutein cells and produce progesterone and oestrogens. Hormone secretion in the corpus luteum ceases within 14 days after ovulation if the oocyte is not fertilised. In this case, the corpus luteum degenerates into a corpus albicans is a whitish scar tissue within the ovaries. Hormone secretion continues for two to three month after ovulation if fertilisation occurs. The different stages of CL are helpful in identifying the function and days of ovulation. In this publication, the author just explained the important corpus luteum in maintaining the ovulated oocytes until fertilization and after formation of zygotes. So there is no evidence on the impact of corpus luteum presence to the ovary and other follicles.

Kumar A. *et al.* (1996) stated that by collecting ovaries from slaughtered buffaloes to study follicular population and compare methods of oocyte retrieval. From their study, they proposed different sized follicles in relation to oocyte diameter ovaries that were studied histologically. They also found that the yield of oocytes per ovary were less ($P < 0.05$) from ovaries bearing a corpus luteum (CL). This study shows that, the effect of corpus luteum on the number of oocytes (follicles) retrieved. This is evidence on the work that has been done by Nur Farah Atiqah Y. in 2015 on retrieving oocytes from slaughterhouse ovaries. Even though they stated the finding on oocyte yield, but no perfect histological studies has been done. So therefore, systematic histological analysis should be done to identify the effect of corpus luteum and the yield of oocytes retrieval.

2.1.3 Follicle

Akers and Denbow (2008) explained the follicular development by the publication of Anatomy and Physiology of Domestic Animals in the Reproduction Chapter from page 508 – 511. In this text book they also present some histological photographs of bovine follicular development. The follicular development shows that the earlier stage of the primordial follicles in the ovary and also the development stages of various follicular state. The histological images gave deeper pictures on identifying the follicles and follicular developing stages within the ovaries. Figure 2.1.3 shows the HE stained follicular image of ovary.

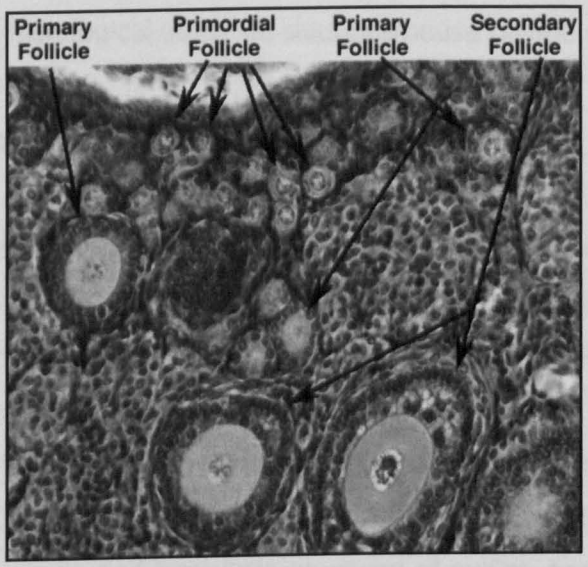

Figure 2.1.3 HE image of follicles

Source: Ansci.Wisc.Edu

Rodgers and Irving (2010) stated that follicle classification is an important aid to the understanding of follicular development and atresia in a ovarian follicular development. Some bovine primordial follicles have the classical primordial shape, but ellipsoidal shaped follicles with some cuboidal granulosa cells at the poles are far more common. Preantral follicles have one of two basal lamina phenotypes, either a single aligned layer or one with additional layers. Besides that, the diameters of the follicles should be considered in classifying the follicles.

2.2 Slaughterhouse Animal (Cow)

Alkire (2009) reported that the culled animal will be send to slaughterhouse for slaughtering. The meat and eatable organ and parts will be sold. The unwanted parts and organ will be threw away or make into meals for other animals. From this article, we can know that, unwanted internal organs are being thrown away. One of the unwanted parts is reproduction organs of female (cow). Even though the cow is been slaughtered but the ovary is still full of potential oocytes. Proper collecting and retrieving of those ovaries with oocytes will helpful in producing IVEP cattle. In Malaysia it is against the law of slaughtering the productive cows. Here we are permitted to slaughter the reproductively unproductive cows. So if that is the situation,

we should analyse the potentiality of those ovaries from the unproductive cows for IVEP use. Therefore histological study on slaughterhouse bovine ovaries is suggested.

2.3 Collection of ovaries from slaughterhouse cattle

Forty (40) repeat breeder beef cows ranging from 5 to 10 years of ages palpated open and were sent to slaughter. The cows belonged to a ranch in southern Florida totalling 10,000 heads of European breeds. The reproductive tracts of the cows were collected, grossly examined, and tissue samples from ovaries, oviduct, uterine body, uterine horns, liver and kidney were taken for histological evaluation, which proposed by Buergelt *et al.* (2010). In this study they proposed that histological method is suitable in identifying the dysplasia and neoplasia as cause of culling beef in Southern Florida. This is shows that, histology is very much useful and also can find that, they also use slaughterhouse ovaries in fulfilling their studies.

Bovine ovaries were obtained from young non-pregnant cycling heifers less than 10 min after slaughter was reported by Vigne *et al.* (1994). This study was conducted on characterization of bovine ovarian surface epithelium and stromal cells: identification of secreted proteins. The methods of collecting and analysing were explained. This would be easier in performing my histological work that starts with ovary collection.

Fry *et al.* (1997) reported that bovine ovaries were collected from local slaughterhouses over a 2 hours and transported to the laboratory in a thermos containing warm (30 to 35 °C) 0.9 % saline solution. They were then rinsed twice with the saline solution and kept at 39 °C until aspiration within 6 hours of collection. This work that they had done is mostly on oocyte collection process. In this study, the important part is the collection of ovaries and the initial handling of the ovaries before the retrieval process of oocytes. Saline solution is usually use in keeping the oocytes alive and the tissues to stay fresh. In histology study this is not that much important in keeping the oocytes and the tissues alive.

Ovaries were dissected out from cattle within 30 to 60 minutes of slaughter, and transported to the laboratory within 2 hours to 4 hours in freshly prepared normal saline solution fortified with 100 IU/ml Benzyl penicillin (Alembic Ltd. Vadodara, India) and 100 µg/ml Streptomycin sulphate (Alembic Ltd. Vadodara, India) maintained at 36

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