

Potential of embryo production techniques in vitro for improving bali cattle seedstock

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Potential of embryo production techniques *in vitro* for improving Bali cattle seedstock

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Abstract. The facts in the field show that seedstock of Bali cattle in South Sulawesi have decreased because the high rate of female productive Bali cattle were slaughtering and good bull maintained for fattening and trading and the price of cow Bali were more cheaper than bull. Therefore, it needs a new breakthrough to solve this problem by applying the embryo production technology *in vitro*. This process consists of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). These three processes have been studied using Bali cattle oocytes, the maturity level of Bali cattle oocyte reaches 91.53%, while the fertilization and division rate was 68%-70,03% and embryo *in vitro* culture of Bali cattle reached 32 cell blastocyst stages. The results of the study indicate that the potential of *in vitro* embryo production can be used to maintain and improve the genetic quality of Bali cattle.

1. Introduction

Research on *in vitro* embryo production in various cattle breed has been carried out in exotic breed such as in Fries Holland, Friesian and Ongole etc, but his research on the Bali cattle is still very limited. The application of embryo production *in vivo* and *in vitro* in Bali cattle still needs study, but it needs to be developed to accelerate population increase and the quality of genetic of Bali cattle. In our laboratory, has conducted research on embryo production *in vitro* for the past 3 years. This is based on adequate facilities and infrastructure and the results of research in the field indicate that the seedstock produced by the breeding program only produce Bali class 1 cattle based on SNI for Bali seedstock, namely <30% [1]. So that needs a new breakthrough by applying *in vitro* embryo production technology (IVEP). The technology of embryo production in this laboratory has several advantages compared to *in vivo* embryo production technology. First, IVEP can be used in problem cows such as cows that fail to respond to superovulatory treatment. Secondly, IVEP can be used to save the genetic potential of sick cows that are not expected to respond the conventional embryo production. Third, semen from different bulls can be used to fertilize the oocytes harvested from the ovary of a female cow which produces a lot of oocytes and potentially fertilized by sperm from various cattle into embryos. Fourth, oocytes for IVEP can be obtained from live donor ovaries using ovum pick up (OPU) or from ovaries of slaughtering cattle [2]. In developing countries such as Indonesia, there is still a high slaughtering of female cattle in the abattoir and many ovaries wasted, the ovarian waste is one alternative to get progressively developing oocytes. This IVEP process consists of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). The technology of *in vitro* embryo production has been investigated using Bali cattle oocytes in the past three years as an

efficient alternative to in vitro systems to produce embryos for propagation faster than germplasm and for research in developmental biology and developing biotechnology in the past decade. In this paper, we will discuss some of the results of research on the three processes of embryo production in vitro in Bali cattle and the opportunity to implement in vitro embryo production technology to improve the genetic quality of Bali cattle.

2. Oocyte Bali on *in vitro* maturation

Application on in vitro technology for improve the quality of Bali cattle embryos begins with invitro oocyte maturation (IVM). Oocytes obtained can originate from the ovaries of ovum pick up (OPU) or from female cattle that are slaughtered in abattoir, although it has a different level of diversity. In research in our laboratory, the oocytes used in the study come from abattoirs. The Factors for character an important role in the success of oocyte maturity in vitro are oocyte quality, maturation time and culture medium used. The success of oocyte maturation is largely determined by the quality of oocytes at the beginning of the oocyte maturation process.

The success of oocyte maturation is largely determined by the quality of oocytes at the beginning of the oocyte maturation process. Oocytes under natural conditions (in vivo) after being ovulated, are in the Germinal Vesicle (GV) stage after the LH hormone has reached the highest level. Oocytes in the GV stage will then develop into the stage of GVBD, Meiosis I and Meiosis II. After the ovary was sliced, an oocyte collection is carried out and further evaluation and selection of oocytes is carried out. Oocyte selection that is widely used is the selection of oocytes based on the morphology of cumulus cells that are around the oocyte [3]. Another criterion that is also often used as an indicator of maturation is the cumulus cells which are assessed based on cumulus cell expansion (fig. 1). Oocytes that want to be evaluated at the stage of development of the cumulus cells are denuded to facilitate coloring (fig. 1C).

This criterion is often used because there is a strong indication of the dynamics of expansion of cumulus cells in oocytes from certain animals with normal morphology, ability to fertilize and the ability of oocyte development after fertilization [5]. The nucleus maturation process is related to RNA synthesis activity, characterized by nucleus changes from the diploten phase to metaphase II. The nucleus membrane will unite with the vesicles forming the vesicle germinal (GV), GV is characterized by the presence of a round nucleus with a complete membrane and filamentous chromatin (fig. 2 A), then the nucleus membrane will be released to form the germinal vesicle breakdown (GVBD). GVBD is characterized by thick chromatin and the absence of a nucleus membrane. After GVBD is formed, the chromosomes are wrapped by microtubules and microfilaments which greatly affect the success of meiotic division. Oocytes that have experienced GVBD will then reach the stage of metaphase I (MI). MI is characterized by the presence of chromosomes that line the equator, the core membrane is no longer visible. (fig. 2)

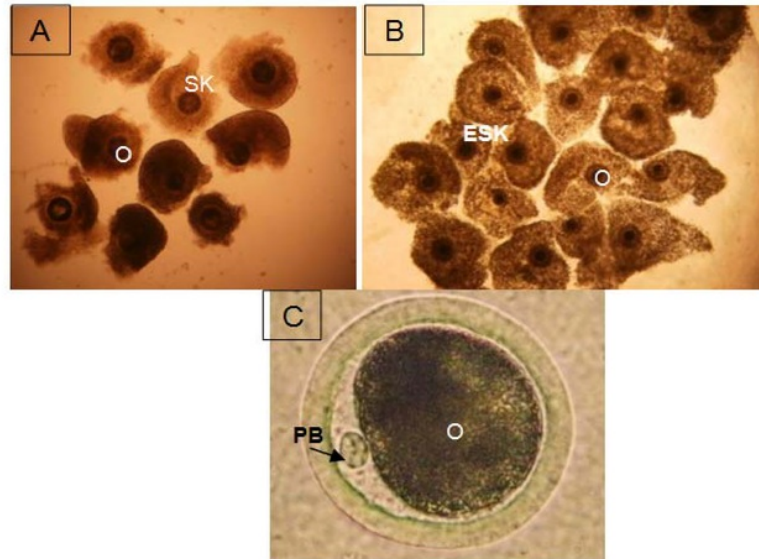


Figure 1. Condition of Bali cow oocytes before maturation (A); after maturation (B) and the condition of denuded thermocytes (C). Description: O = oocytes, SK = cumulus cells, ESK = Cumulus cell expansion, PB = Polar body. 40x magnification [4]

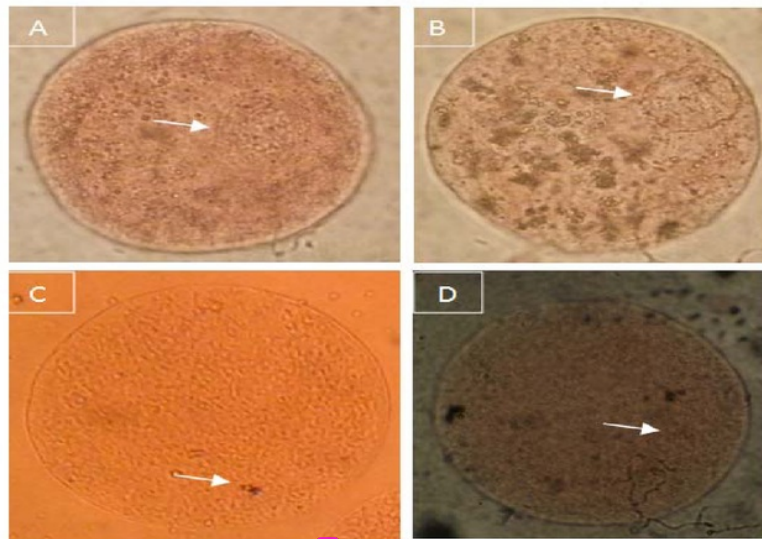


Figure 2. Level of Oocyte Core Maturation. A. Germinal vesicle (GV); B. Germinal vesicle breakdown (GVBD); C. Metaphase I (MI); D. Metaphase II (MII). 40x magnification [4]

Cytoplasmic maturation is characterized by a number of criteria including cytoskeletal organization of oocytes such as cortical granular migration to oolemma, increased mitochondria and lipid droplets, changes in the Golgi apparatus and the presence of granular endoplasmic reticulum, MPF activity and oocyte metabolism [6]. Core ripening can be evaluated by staining such as aceto orcein [7], while cytopathic maturation can be identified indirectly, among others, from cortex reactions, pronucleus formation, and cell division [8].

Based on the quality of the oocytes Bali cows are grouped into 4 consisting of four, namely quality oocytes A, B, C and D according to the appearance of cumulus cells and cytoplasm of the oocytes themselves [9]. Ovarian status is divided into groups namely 1) ovaries with CL (corpus luteum) and DF (dominant follicles), 2) ovaries without CL and there are DFs, 3) ovaries with CL and without CL, and 4) ovaries without CL and DF. The results showed that the number of oocytes that reached the metaphase II (MII) phase was higher ($P < 0.01$) in the ovary pair with CL and without DF (89.47%) compared to those without CL and without DF (75.47%), without CL and there was DF (74.41%), and there was CL and there was DF (65.52%). Ovarian reproductive status that has CL but without DF has the highest number of oocytes reaching the MII stage.

The medium used for oocyte maturation can have an effect not only for the oocyte maturation process but also for embryo development. Based on the composition of the constituent materials the medium commonly used for embryo production processes in vitro can be divided into simple mediums such as whitened medium, brinster medium, whitened dan biggers, human tubal fluid (HTF), chatot ziomex bavister (CZB), dan potassium simplex optimized medium (KSOM), a complex medium such as Ham's F-10 and tissue culture medium (TCM) 199 and sequential medium such as G1 and G2 [10]. Hasbi et., al [11] reported that insulin-like growth factor-I (IGF-I) of 100 ng/ml was added to the maturation medium and effectively increased the oocyte to achieve maturity of the metaphase II (M-II) stage by 87.5%. In addition to the medium of time it can also affect the level of oocyte maturity in Bali cattle. Sonjaya et al [4] study states that the best maturation time in Bali cattle embryo production in vitro is 28 hours with the number of oocytes reaching metaphase II stage is 91.53%. (table 1). The results of this Bali cattle study are relatively the same as Suthar's research, (2008) which resulted in 94% oocyte maturation rates from Sahiwal x Holstein Friesland crossing with the Opum Pick Up (OPU) method.

Table 1. The maturation level of Bali cattle oocytes nucleus with different maturation times

Turation Time	Oocyte Number	Rate of nukleolus maturation (%)			
		GV	GVBD	MI	MII
24	50	-	-	24.07±6.45 ^a	75.93±6.45 ^a
26	50	-	-	19.93±1.15 ^a	80.07±1.15 ^a
28	48	-	-	8.47 ± 0.56 ^b	91.53±0.56 ^b
30	43	-	-	21.37±8.14 ^a	78.63±8.14 ^a

Description: GV = vesicle germination, GVBD = germinal vesicle breakdown; MI = metaphase I; MII = Metaphase II. The same superscript in the same column shows the data is significantly different ($P < 0.05$)

In general, the maturation medium is supplemented with gonadotropin hormones (follicle stimulating hormone / FSH and luteinizing hormone (LH) and estradiol 17 β which is reported to significantly increase the oocyte maturation rate [13]. The addition of gonadotropin hormones in the maturation medium is reported to increase oocyte quality and development ability with possible changes in metabolic processes [14]. Gonadotropin hormone is the main regulator for the maturation of oocyte nucleus in mammals in vitro. Estradiol may be involved in cytoplasmic maturation by stimulating DNA polymerase β and is thought to increase the synthesis of male pronucleus growth factors. The presence of estradiol 17 β on oocyte maturation can significantly increase blastocyst production [15]. In addition, serum is also often added to the maturation medium because the serum contains unidentified growth factor, hormones and peptides that might support oocyte growth and development. The serum provides nutrients for cells in COCs and prevents hardening of the pellucular zone (ZP) in sheep oocytes [16]. In the maturation Bali cattle oocytes, we collected oocytes by

slicing technique in the Phosphate Buffer Saline (PBS) medium supplemented with Fetal Bovine Serum (FBS) 10% and penicillin streptomycin 100 IU / ml. Oocytes are matured for 24 hours, 38.5 °C in a 5% CO₂ incubator, using TCM-199 medium supplemented with FBS 10%, Follicle Stimulating Hormone (FSH) 10 IU / ml, Luteinizing Hormone (LH) 10 IU / ml, and gentamycin 50 µg / ml

12 Oocyte fertilization in Bali cows

Fertilization is a complex process that results in the incorporation of spermatozoa and oocytes, as the initial signaling transition from oocytes to embryo [17]. The penetration of sperm into the oocyte will cause the oocyte to complete meiosis II division which is characterized by the formation of the polar body II. Furthermore the oocyte chromosome forms the female pronucleus and chromatin on the head of the spermatozoa experiences condensation and then forms the male pronucleus. Activation of sperm in oocytes will cause an increase in intracellular calcium thereby reducing the activity of maturation promoting factor (MPF) and mitogen activated protein (MAP) kinase. Inactivation of MPF and MAP kinase during fertilization is related to the release of polar bodies II and pronucleus formation [18].

The results of the study in Bali cattle, ovarian status did not affect the level of fertilization of Bali cattle, the value ranged from 55.54 – 70.03% (table 2.)

Table 2. The number of pronucleus and the level of fertilization that is formed in the oocyte of cattle with different maturation times

Duration Time	Oocyte Number	Nukleus Formation				Fertilization rate (%)
		0PN (%)	1PN(%)	2PN(%)	>2PN(%)	
24	56	8 (14.29)	17 (30.36)	26 (46.42)	5 (8.93)	31 (55.54)a
26	49	2 (4.08)	18 (36.73)	27(55.10)	2(4.08)	29(59.73)a
28	50	6(12.00)	9(18.00)	34(68.00)	1 (2.00)	35 (70.03)b
30	51	11 (21.25)	7 (13.73)	30(58.82)	5 (9.80)	33 (64.21)ab

Description: PN : Pronukleus . The same superscript in the same column shows the data is significantly different (P <0.05)

To be able to carry out the fertilization process, the spermatozoa cells must first undergo a process of capacitation. Capacitation is a process of preparation and physiological changes in spermatozoa in the reproductive tract of the female to enhance her fertility. At the time of capacitation, intracellular calcium levels increase, so extracellular calcium is needed. Modulation of Ca²⁺ in spermatozoa from ejaculation may occur through joint action of Na⁺ / Ca²⁺ porter and Ca²⁺ -ATPase. During capacitation there is an increase in intracellular Ca²⁺ resulting from a bali from the antiporter or an inhibition of Ca²⁺ -ATPase [19].

25 4. Bali cattle in embryo culture

In vitro culture (IVC) is the final stage in the application of in vitro embryo production technology after in vitro fertilization (IVF) [24] and in vitro maturation (IVM) [20]. The ability of embryo development after fertilization is influenced by many factors, including the competence of oocytes (the ability to begin the process of meiosis, the ability to divide after fertilization, the ability to divide to the blastocyst stage, the ability to make pregnancy and the ability to develop well and healthy), oocyte maturation [21]. After the invitational fertilization process, the proportion of zygote that can develop into the blastocyst stage during culture is around 30-40% [22]. The results of Bali zygote culture showed that the speed of embryonic cell division on certain days was different, this was indicated by the presence of 2-cell, 4-cell and 8-cell embryos on the second day (figure 3).

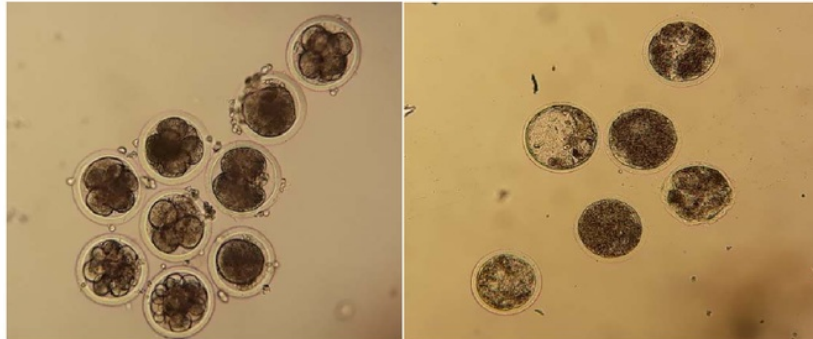


Figure 3. Embryo Development Ability. A: 2-day-old embryo and B: 8-day-old embryo.

The intrinsic quality [22] oocytes and the environment or culture media after fertilization are the main factors that greatly influence the ability of development to the blastocyst stage [3]. It was further explained that embryos [14] are very susceptible to various in vitro stresses including among them can be caused by improper media formulations, supplementary media, problems in culture systems, technical problems or lack of proper quality control and quality assurance. Short-term impacts that can be observed with the presence of in vitro stress include changes in morphology, cell proliferation [16] and apoptosis, metabolism, transcriptome, and proteome, while medium and long-term impacts include low pregnancy rates after transfer, high risk of abortion, length pregnancy, congenital abnormalities, death after birth, and emergence of disease after adulthood.

5. *In vitro* embryo transfer and implications for genetic quality improvement of Bali cattle

Embryo transfer technology (ET) in cattle is the second generation of reproductive biotechnology after artificial insemination (AI). Embryo transfer is a process whereby the embryo is transferred from a female animal acting as a donor at the time the embryo has not undergone implantation, to a female who acts as a recipient so that the prescription becomes pregnant. Recipient livestock readiness plays an important role. Collections and TE can now be carried out in a non-operating manner, so that it will facilitate the implementation in addition to the relatively more economical costs. Successful transfer of fresh embryos can reach 55 - 65%, while frozen embryos are around 50– 60% [23]. The application of embryo transfer technology in Bali cattle was only at the initial stage, of the five Bali cattle recipients, only four were declared pregnant, this initial step needs to be further studied with more embryo recipient numbers.

The application of *In vitro* Embryo Transfer Technology to improve the genetic quality of cattle (fig. 4), can be explained as follows:



Figure 4. Scheme Of In Vitro Embryo Production To Improve Bali Cattle

1. Selection of cows with good productivity as a source of oocytes and males is good as a source of sperm.
2. Oocytes can be collected from live cattle using the technique of "Ovum Pick Up" or originating from the main Ovary cut in the Slaughterhouse.
3. Perform oocyte maturation, fertilization and embryo culture in vitro
4. The embryo can be transferred in the form of fresh or frozen embryos.

On smallholder farms in the province of South Sulawesi, generally the cows' maintenance is still traditional, at the end of the harvest season, female cows grazed in paddy fields are usually combined with small or young males, while large males are generally kept in cages or tied in under house. This has the effect of "inbreeding" which decreases the quality of cow seedstock. In the working season in the rice fields, groups of cattle are grazed in gardens. Artificial insemination did not affect significantly on calving rate and genetic quality cause the traditional maintenance.

6. Conclusion

Ovary of Bali cattle from abattoir after embryo technology in vitro process results in a high level of oocyte maturation, high fertilization and embryo production. The process of in vitro embryo production technology accompanied by breeding techniques in oocyte and sperm sources has the potential to accelerate and improve the genetic quality of Bali cattle

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