The role of cell apoptosis on *in vitro* produced beef cattle embryos

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Declaration

I, Marble Nkadimeng declare that the dissertation, which I hereby submit for the degree MSc. Animal Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other tertiary institute

Signature……
Date……

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Abstract

The *in vitro* culture environment of cattle embryos can compromise the survival of developing embryos resulting in cell apoptosis. Detection of cell apoptosis is important for determining embryonic quality and reducing embryonic mortality in female animals prior to transfer. In this study the role of cell apoptosis on *in vitro* embryos was studied, with the focus on oocyte maturation and embryo production, caspase-3 activity and DNA fragmentation. Cow ovaries were collected from local abattoir and a total of 900 COCs were retrieved per week for the study over five-month period (six replicate/per experiments). COCs were randomly allocated to four incubation temperatures (39, 41, 42 and 43 ˚C) for polar body extrusion. Based on maturation results, two preferred temperatures (39 and 41 ˚C) were selected for maturation. Oocytes were subjected to normal subsequent embryonic conditions post maturation. Embryos produced from both maturation temperatures were then examined for embryonic development, caspase-3 activity and DNA fragmentation for evidence of apoptosis. No difference was ($P<0.05$) observed in embryonic development between oocytes matured at 39 ˚C and 41 ˚C respectively. Blastocysts produced at 39 ˚C showed significantly ($P < 0.05$) higher nuclei cells compared to those produced from 41 ˚C matured oocytes. A higher ($P< 0.05$) caspase-3 activity and DNA fragmentation were observed at (2-4 cell) and (≥ 8cell cell) embryos for 41 ˚C matured oocytes compared to 39 ˚C maturation group. A significantly higher caspase-3 activity and DNA fragmentation on blastocyst produced at 41 ˚C matured oocytes were also evident compared to those produced at 39 ˚C. Although embryos produced from oocytes matured at 41 ˚C showed similar developmental capacity to embryos from 39 ˚C, this study showed that a higher incidence of apoptosis can be expected in embryos produced from oocytes matured at a higher (41 ˚C) temperature than 39 ˚C.

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ART	Assisted reproduction biotechnologies
Apaf-1	Apoptosis protease activating factor-1
Bad	Bcl-2 Associated agonist of cell death
Bak	Bcl-2-antagonist/killer 1
Bax	Bcl-2-associated X protein
Bcl-2	B cell CLL/lymphoma 2
Bcl-XL	B-cell lymphoma -extra large
Bid	Interacting domin death agonist
BO	Bracket and Oliphant
Bok	Bcl-2 related ovarian killer
BSA	Bovine serum albumen
°C	Celcius
COCs	Cumulus oocyte complexes
CO_2	Carborn dioxide
CX 43	Connexin 43 gene
DPBS	Dulbecco phosphate buffer saline
DNA	Deoxyribonucleic acid
DNase	deoxyribonuclease
dsDNA	Double-stranded deoxyribonucleic acid
EGA	Embryonic genome activation
ET	Embryo transfer
EST	Estradiol hormone
FBS	Fetal bovine serum
FSH	Follicular stimulating hormone
G6PH	Glucose-6-phosphatate dehydrogenase
GLUT1	Glucose transporter 1
H_2O_2	Hydrogen peroxide
HSP	Heat shock proteins
HPRT	Hypexanthine-guanine phosphoribosyl transferase
ICAD	Inhibitor of caspase activated deoxyribonuclease
IVM	In vitro maturation
IVM	In vitro culture
IVF	In vitro fertilization

IVP	In vitro embryo production
LH	Luteinizing hormone
LOS	Large offspring syndrom
Mcl-1	Myeloid cell leukemia
MDPBS	Modified dulbecco phosphate buffer saline
MOET	Multiple ovulation and embryo transfer
O_2	Oxygen
OPU	Ovum pick up
PBS	Phosphate buffer saline
PBS-PVP	Phosphate buffer saline-polyvinylpyrrolidone
ROS	Reactive oxygen species
RPM	Revolution per minute
SOF-BSA	Synthetic oviductal fluid- bovine serum albumen
SOF-FBS	Synthetic oviductal fluid - fetal bovine Serum
TDT	Terminal teoxynucleade tansferase reaction
TCM	Tissue culture mediu
TP53	Tumour protein 53

Chapter 1

1.1 . **Introduction**

Reproductive inefficiency is one of the most important causes of economic losses in livestock industries and it is realized throughout the world (Verma *et al.*, 2012). Therefore, there is a need for application of reproduction biotechnologies in livestock to aid in animal improvement. Reproductive biotechnologies are useful for solving possible reproductive failures. Thus, Assisted Reproductive Technologies (ART) have been developed and refined to obtain large number of offspring from genetically superior animals or obtain offspring from infertile or subfertile animals (Widayati, 2012). The first biotechnology procedure used to improve reproduction and accelerate genetic improvement of livestock was artificial insemination (AI), followed by MOET (Multiple Ovulation and Embryo Transfer) and *in vitro* fertilization (Niemann & Kues, 2003). Development of reproductive technologies such as; estrus synchronization, superovulation, non-surgical embryo collection, and cloning to name a few are more recent assisted reproduction technology tools used in improvement of livestock production (Hafez, 2015). However, the common goal of all the biotechnologies is to provide highly reliable mechanism to produce superior livestock which can help animals of average productivity (Jennifer *et al*., 2013). These technologies (AI, MOET and IVP) have been evolving throughout the years based on their effectiveness with regards to male and female fertility.

The AI technology maximizes the use of outstanding males by dissemination of superior genetic material, improve the rate and efficiency of genetic selection and introduction of new genetic material by import of semen rather than live animals. A large number of AIs are performed globally; it is estimated that more than 100 million cattle, 40 million pigs, 3.3 million sheep and 0.5 million goats are artificially inseminated every year (Boa-Amponsem & Minozzi, 2006; Verma *et al*., 2012). The first successful insemination was performed by Spallanzani (1784), on a bitch (Verma *et al*., 2012). It was through the success of Spallanzani where various aspects of AI technology have been globally standardized for each species. Smith (1988), introduced the concept of MOET and demonstrated how well designed MOET programmes could lead to increased selection intensity and reduced generation intervals, resulting in improving genetic gain (Chakravarthi *et al*., 2012). Genetic contribution of both male and female gametes can be used at the same through embryo transfer technology. Thus this improve livestock at a faster rate (Hafez, 2015).

Moreover, data retrieved from the International Embryo Transfer Society committee report estimated the number of embryo transferred to be 800,000 in cattle, 25,000 in sheep, 7000 in goat, 30,000 in pig and 12,000 in horses worldwide with a conception rate of 55-70% (Hafez, 2015).

Embryo biotechnology is one of the powerful tools for animal breeders to improve genetic progress of their livestock herds (Wu, 2012). The objectives of genetic improvement of livestock include the acceleration of genetic progress often with the focus on a specific trait (Wu, 2012). Over the past three decades, embryo biotechnology has evolved through three stages: firstly, by superovulation which involves retrieval of embryo derived from donors and non-surgical recovery and transfer of cattle embryos, secondly, *In vitro* embryo production by *in vitro* fertilization by ovum pick up to retrieve oocytes and thirdly subsequent *in vitro* technologies, particularly cloning by somatic cell transfer, embryonic stem (ES) cell development, transgenic animal production and embryo transfer (Betteridge, 2004; Lonergan, 2007).

In vitro embryo production (IVEP) is one of the most crucial techniques amongst embryo biotechnologies used in farm animal breeding since it does not only help in production of high genetic merit animals, but also serves as an excellent source of embryos for emerging biotechnologies like embryo sexing, cloning, nuclear transfer and transgenesis (Duszewska *et al*., 2012; Hafez, 2015). Furthermore, it allows analyzing embryo development potential, including the pattern of gene expression, epigenetic modifications and cytogenetic disorders during the development (Galli & Lazzari, 2008). The technology generally refers to a number of procedures performed in the laboratory which includes maturation, fertilization, and culture procedures required to produce embryos from immature oocytes (Duszewska *et al*., 2012). The first successful IVF was achieved on rabbits in 1959 (Chang, 1959), followed by mice in 1968, human in 1978 (Steptoe, 1980) and the first born calf produced with IVF was in 1981 (Brackett *et al*., 1982).

Handling of gametes *in vitro* however will always impose a threat to cells as they are under artificial conditions and will be easily damaged. The biological and practical reasons for damage might be the IVM Media compositions, *in vitro* handling in a dish, working conditions e.g. distance between oocyte donors or slaughter house and IVM laboratory, temperature variation and oxygen levels in the environment where embryos are handled (Callesen, 2012). However, the effect of the damage depends on the quality of the embryos or the techniques itself. Some embryos are more resistance to the stresses than

others. It is therefore important to realize the extent of the damage and evaluate its importance (Brad *et al*., 2007; Callesen, 2012).

Gene expression patterns are one of the differences between *in vitro* produced embryos with the *in vivo* embryos in farm animal studies. *In vitro* embryos results in low expression of fundamental genes (Lonergan & Fair, 2008; McHughes *et al*., 2009) due to cell apoptosis caused by its culture environment. Techniques are however available for morphological embryo quality evaluation such as total cell number determination by nucleus staining using propidium iodide (PI) or Hoechst (Pursel *et al*., 1985). However, in the South African laboratory systems, apoptotic assessment of embryos has not yet been evaluated. Apoptosis is defined by Fear & Hansen (2011) as the "self-destruction of cells under physiological control". Apoptosis is one of the major aspects caused by imbalance of genes and culture environment of *in vitro* embryo production. It is characterized by cell shrinkage, translocation of phosphatidyl-serine to the outer cytoplasmic membrane, DNA fragmentation, and segmentation of the cell into apoptotic bodies (Elmore, 2007). Apoptosis is regulated by members of the Bcl-2 gene family such as the pro-apoptotic Bax and anti-apoptotic Bcl-2 and occurs through activation of caspases, a family of cysteine proteases. The activation of the Bcl-2, specifically the pro-apoptotic member genes is performed by the release of cytochrome C which interact with the apoptosis enzyme Apaf-1 and therefore activate caspase 9. The other activation of the anti-apoptotic member of the Bcl-2 gene is through the blocking apoptosis induced by the external stimulus (Ebrahimi *et al*., 2010) and when the the pro-apoptotic protein BAX is expressed it counteracts the apoptosis-preventing effect of BCL-2 (Somal *et al*., 2015).

Activation of these caspases releases DNAse enzymes that breaks the 3' OH end of the DNA which later causes DNA fragmentation. This will then trigger the release of apoptotic initiator gene, the Bax gene. (Gjørret *et al*., 2003). The significance of evaluating the role of apoptosis caspases on *in vitro* embryos is an important initiative as this may lead to new methods for improving *in vitro* embryo production systems through selection of healthy embryos. It can be useful in developing molecular signatures to improve morphology-based embryo evaluation. This could further provide an understanding and aid in reducing embryonic mortality in female animals to improve implantation rates in cattle breeding. Moreover, there is no information on apoptosis evaluation of cattle in vitro embryo production in the South African context of beef cattle breeds. Therefore, assessment of apoptosis will aid in improving the efficiency of the IVEP prior evaluation and for better implantantation purposes.

1.2 . **Justification**

In South Africa, the use of reproductive technologies such as AI in beef cattle is most popular in stud cattle breeding, although AI is used on a limited scale in three-way crossbreeding systems to improve the management of the breeding program (van Marle-Köster & Webb, 2014). However, *in vitro* fertilization and embryo transfer in South Africa has been used more in wildlife compared to cattle breeding to breed disease-free buffalo and other scarce African ungulates such Roan and Sable antelope, due to the increasing monetary value of these species for the game and hunting industry (van Marle-Köster & Webb, 2014). The use of IVF embryos by cattle breeders however has been steadily growing in South Africa over the years for building breed numbers and increasing genetic progress. During the past breeding seasons, production of embryos has increased from 2,858 in 2010, 5,028 in 2011 to 6,384 in 2012 through IVF (Invitrobrasil, 2016). The use of this technologies could lead in protein production needed by the estimated 9.5 billion growing population by 2050 (Thornton, 2010) in developing countries due to its potential of increasing the number of offspring per cow to up to 100 offspring in a year.

However, it has been documented that the *in vitro* culture (IVC) environment causes low expression levels of genes in cattle *in vitro* embryo production (IVEP). The IVC causes mitochondrial death, triggers stress activators that results in early embryonic loss due to cell apoptosis. This further leads to oocytes incompetence, chromosomal abnormalities and large offspring syndrome (LOS) (Farin *et al*., 2010). It is therefore important to evaluate the IVP system in terms of cell apoptosis for healthy embryo production *in vitro*.

1.2.2*.* **Aim of study**

The aim of the study was to evaluate the role of cell apoptosis on *in vitro* beef cattle embryos, with focus on oocyte maturation and embryo production, caspase activity and DNA fragmentation. The aim was achieved by the following objectives:

- To investigate the extrusion of polar bodies following four maturation temperatures: 39, 41, 42 and 43°C on cattle oocytes, and subsequent *in vitro* embryo production from cattle oocytes matured at 39 and 41 °C.
- To investigate the caspase-3 activities on *in vitro* produced embryos following 39 and 41°C incubation temperature at different stages of cattle *in vitro* embryo production.

- To investigate DNA degradation on *in vitro* embryos using tunel assay method from both 39 and 41°C incubation temperature at different stages of cattle *in vitro* embryos.

Chapter 2

Literature review

2.1. **Introduction.**

In vitro embryo production (IVP) technology holds the potential to increase the number of offspring per cow to up to 100 offspring in a year. It can therefore contribute to a higher acceleration intensity in the herd and increase genetic progress (Suthar & Shah, 2009; Vilarino & Ross, 2015). In the beef cattle industry, IVP is used to develop new breed lines for increased selection, higher quality beef products or disease resistance and increase growth rate (Greger, 2010). Moreover, the use of IVP in the beef industry has been implemented largely by pure beef breeders (Wu, 2012). However, the implementation of the technique poses a challenge world-wide to different reproduction specialists due to differences in the artificial environment of the growing embryos. The artificial embryo environment in most cases lead to apoptosis or cell death of the developing embryos. This chapter therefore focused on the review of available literature on *in vitro* embryo production technique, its effect, limitations, genetic regulations and apoptotic effects due to the *in vitro* environment of beef cattle embryos.

2.2. **Overview of *in vitro* embryo production in the beef cattle industry**.

The gestation period of a cow results in one offspring per year and thus tends to narrow the selection processes. However, assisted reproduction technologies (ART) can be used to improve selection and distribution of high genetic merit animals in the beef cattle industry (Kadarmideen *et al*., 2015). The ART procedures that can be applied to enhance reproduction in this industry are fertilization of oocytes either *in vitro* through collection of oocytes or *in vivo* through artificial insemination, embryo recovery through uterine flushing, *in vitro* embryo production and transfer of embryos to surrogates (Wu, 2012). *In vitro* embryo production through its processes such as oocytes maturation, fertilization and subsequent embryo production allows more frequent gametes collection and therefore further increase the number of transferable embryos (Plourde, 2012). Additionally, it has been mention that in *vitro* embryo production technique is estimated to yield three times more embryos than other ARTs through the constant collection of oocytes (Plourde, 2012). Retrieval of immature oocytes from cattle for initialising IVP can either be achieved from slaughter house ovaries or from a live donor cow. Ovum Pick Up (OPU) is one of the techniques used for retrieval of oocytes from a living donor in cattle. This technique was design for practical application where it was desirable to recover oocytes from living donors of known genetic value. However, according to Gallia *et al*. (2014), all the experimental work done to develop *in vitro* embryo production (IVP) procedures

was essentially based on oocytes that were recovered from slaughter house ovaries in large numbers.

Since *in vitro* embryo production has been more successful as shown in figure 2.1, according to the International Embryo Transfer Society (IETS) statistics, the number of embryos produced *in vitro* and transferred into recipients has increased more than 10 times in the last decade and are now approaching the numbers of embryos produced *in vivo* by superovulation (Gallia *et al.,* 2014; Mapletoft, 2015). This indicates that IVP is considered a reliable and cost-effective technique and deserves a role in cattle breeding (Gallia *et al.,* 2014). *In vitro* production of embryos is steadily growing and the majority of them are now produced by ovum pick up (OPU). The tremendous growth of IVP is shown on figure 2.1.

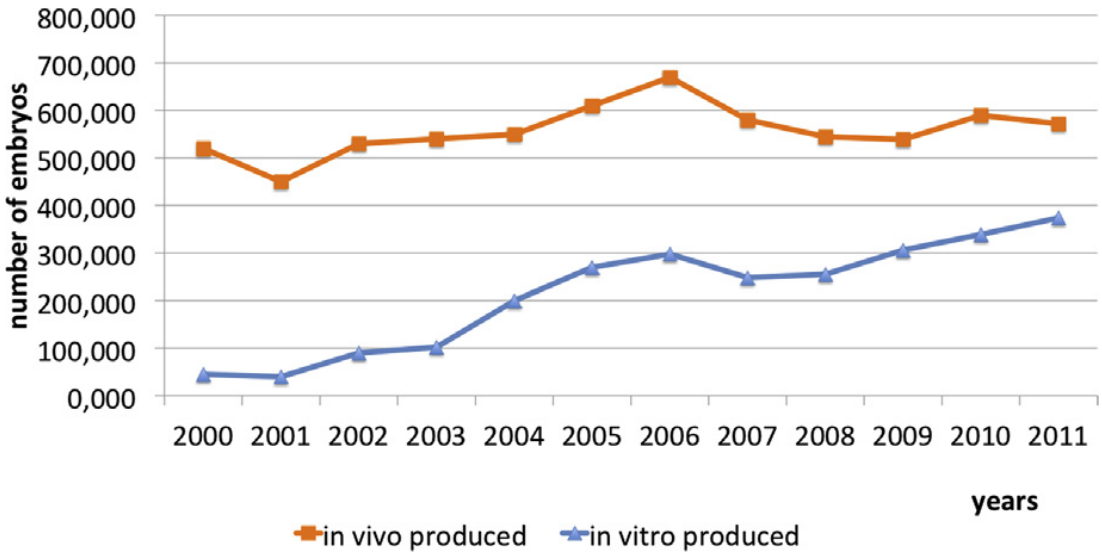


Figure 2. 1 Summary of the international Embryo Transfer Society stastistical data collected by the data retrieval committee (Gallia *et al*., 2014).

Authors, Pfeifer *et al.*, (2008) and Machatkova *et al.,* (2008), stated that IVP is an alternative that can be applied to accelerate genetic improvement via production of a larger number of embryos. This will therefore be vital for commercial usage, selection of high value donor cows, production of transgenic animals and embryo sexing. However, Paula-Lopes *et al*. (2012) and Hansen (2006) mentioned important technical limitations in IVP which collectively includes increased costs, sub-optimal embryonic, foetal survival and occasionally abnormal offspring known as large offspring syndrome. All above mentioned aspects are set to reduce the desirability of *in vitro*-produced embryos (Hansen, 2006). Plourde (2012), however, suggested that in *vitro* embryo system should be evaluated for an improved understanding of potential deviations.

Principles of in vitro embryo production

The IVEP generally refers to a number of procedures performed in the laboratory that includes oocyte maturation, fertilization, and embryo culture steps required to produce embryos from immature oocytes (Blanco *et al.,* 2011). The IVEP is set as an alternative to the *in vivo* embryo production by superovulation, also known as conventional embryo transfer (ET) (Viana *et al*., 2010). IVEP technique begins with: *in vitro* maturation (IVM) followed by in *vitro* fertilization (IVF) and *in vitro* culture (IVC). Maturation of mammalian oocytes as defined by Blanco *et al*. (2011), is the number of steps taking place from the germinal vesicle stage to completion of the second meiotic division with formation of the second polar body. IVM starts with retrieval of oocytes known scientifically as female germ cells either by OPU from a live animal or by retrieval from ovaries of a slaughtered animal. The oocytes are arrested at the early diplotene phase of meiotic prophase I. These cells are initially grouped within large, interconnected clusters known as oocytes nests. During the meiotic arrest of an oocytes the primordial follicles assembles, thus the processes is known as follicular assembles. Moreover, There's recruitment of primordial follicles into a growing cohort identified as primary follicles. Both processes define the earliest stages of folliculogenesis and are believed to affect the period of the female reproductive life span (Skinner, 2005; Picton *et al*., 2008).

Folliculogeneis is known to be one of the important procedures on the release of quality oocytes. The early events of production of oocytes is mostly regulated by steroid hormones that are predominant in ovarian biology such as progesterone, estrogen and androgen. Proper activating of folliculogenesis allows the growth of primordial follicles. During improper activation of follicologenesis other primordial follicles remain dominant for some time depending on the type of specie and this can therefore delay the maturity of the oocyte (Skinner, 2005). Reproductive disorders such as large offspring syndrome can intervene on proper follicular transition in cattle and sheep. Proper activation of folliculogenesis is important as it determines nuclear and cytoplasm maturation of the oocyte (Sirard, 2001). Furthermore, it's only a good quality oocyte that can be able to sustain embryo development prior transfer (Brevini-Gandolfi & Gandolfi, 2001).

Once oocytes are matured, it is critical to introduce the oocytes to sperm that have already been capacitated or are undergoing capacitation for fertilization. Capacitated sperm usually refers to biochemical modifications that allow the acrosome reaction upon exposure to the zona pellucida, cumulus cells, or other substances associated with *in vitro*–matured or ovulated oocytes (John & Parrish, 2013), the process therefore is referred to *in vitro* fertilization. The last stage IVP is known as *in vitro* culture. That is the development of the

embryos from zygote to the morula and blastocyst stages (Duszewska *et al*., 2012). According to different authors (Lonergan & Fair, 2008; Paramio, 2010; Dang-Nguyen *et al*., 2011), this encompasses nuclear and cytoplasmic modifications and their mutual interactions. Moreover, it further refers to changes in embryo metabolism, embryonic genome activation, modifications of gene expression, formation of the morula and compaction of blastomeres. It also formation of the blastocyst with a characteristic structure named the blastocyst cavity that arises as a result of cavitation, and formation of additional structures such as the capsule in the horse embryos.

The expression pattern of specific genes according to Wrenzycki *et al.* (2004), can aid in identifying molecular marker during immature oocyte and during the pre-implantation period. This will aid in identifying the molecular loophole in IVP. One of the factors that can affect the success of the *in vitro* system is apoptosis and large offspring syndrome (LOS) (Hill, 2014) and apoptosis (Yang & Rajamahendran, 2002). There are however studies that provides information of gene expression on genes such as Cx43, HSP, G6PH, Oct-4, HPRT and GLUT-1 during oocytes maturation and embryo development (Dalbies- Tran & Mermillod, 2003), however, the expression of these genes can undergo changes and its effect on the expression changes is still unknown (Lonergan *et al.,*2003a).

The micro-environment of *in vitro* embryos production requires maintenance at all times for the success of embryo development (Rivera *et al.,* 2004a; Jin *et al*., 2007). Factors such as heat or cold shock are common in disrupting the success of embryos development and survival prior transfer in artificial environment of *in vitro* embryos (Edwards & Hansen, 1997; & Wan *et al*., 2015). Furthermore, *in vitro* embryo needs to adjust to its own physiology in order to survive external stresses or it must have the ability to use the maternal information to restore its favourable micro-environment. According to Al-Katanani *et al*. (2002), production of heat shock protein can assist the embryos to adjust to its own physiological environment during external stress for survival (Al-Katanani *et al*., 2002). The quality of embryos depends on the correct amount and time of expression of fundamental genes during oocytes maturation, fertilization, first cleavage and embryo genome activation (Hansen, 2009). Therefore, the genetic information received from maternal (oocyte) and paternal (sperm) is set to be the first most crucial step to determines the quality of the embryo during embryo production (Hwang *et al*., 2010).

Environmental factors influencing in vitro embryo production

Several negative impacts have been reported for IEVP that can be attributed to its artificial micro-environment (Côté *et al*., 2011). Oxidative stress during the past decade has also been

identified to affect the artificial micro-environment of embryo production resulting in either apoptosis or abnormal embryonic development. Furthermore, stress can impair mitochondrial integrity and its activity. Oxidative stress occurs as a result of an imbalance between antioxidants and reactive oxygen species (ROS) production. During *in vitro* culture of embryos, endogenous and exogenous factors are known to induce oxidative stress and temper with the balance of antioxidants. Moreover, the ideal oxygen tension for embryo culture is 5% (Yuan, 2003; Laura *et al*., 2007). Decrease ROS production and improved embryo metabolism tend to be associated with low oxygen levels in embryo production, thus this increase embryo production. (Ahmed *et al.*, 2012).

The micro-environment in which the oocyte grows and mature defines its viability. Following oxidative stress, the most common environmental determinant in IVP of oocyte viability is heat stress. Heat stress alters secretion of hormones such as LH (luieternizing hormone), FSH (follicular stimulating hormone) and inhibin involved in follicular function. According to Paula-lopes & Hansen (2002), heat stressed oocytes reduce the ability for fertilization and therefore compromises the developmental stages of the embryos, either from fertilized or chemically activation.

Moreover, it is evident that heat stress affects growth of the follicles 30-40 days before oestrus during experiments evaluating damage by heat stress on follicular and oocyte function (Brad *et al*., 2007 and Paula-Lopes *et al*., 2012). The oocyte remains susceptible to heat stress damage until nuclear and the cytoplasmic maturation is completed (Ju *et al*., 2005). According to Hansen (2009), heat stress causes a reduction in the proportion of oocytes that can become normal embryos later in development when applied 10h beginning of oestrus. During maturation of oocytes, heat shock further induces disruption in chromosome alignment, decrease the ability of the oocyte to reach metaphase II. Therefore, the environment in which is ideal for IVP is of most important as it determines the future produced embryo.

2.3. **Genetic regulation and action of *in vitro* embryo production.**

The production of embryos *in vitro* firstly depends on the production of maternal RNA and synthesis of proteins for the maintenance of the oocyte before fertilization. The mRNA synthesises proteins for embryo survival until the 4 cell stage in cattle (Badr *et al.,* 2007). Furthermore, as production proceeds prior the 4 cell stage, RNA and proteins degenerate and the embryo will then depend on the newly gene transcript (Memili & First, 2000). In embryo development this is characterized as minor bovine embryonic genome activation, and is the

process by which the embryo development depend on its maternal transcript for survival until the activation of major embryonic genome starts, which occurs at the 8 cell embryonic stage (Graf *et al*., 2014). In cattle, the zygotes and embryos become transcriptionally and translationally active between 1 cell to 4 cell stages. The process by which the first transcripts are active prior 4 cell stage in cattle is called minor genome activation (Barnes & Eyestone, 1990; Jiang *et al*., 2014). In oocyte maturation and early embryo cleavage (1-4 cell), the maternal transcripts and proteins synthesised are used to sustain the embryo until major genome transcripts are produced (Dieleman *et al.*, 2002; Lee *et al*., 2014). The maternal mRNAs after fertilization, prior to first cleavage, supports the successful initialization of embryonic genome activation (EGA) (Kanˇka, 2009).

Once the mRNA has degenerated, the embryo is ready to switch from maternal to depend on the embryo transcript for survival, therefore fundamental genes will be produced. The RNA and the proteins from the maternal cease due to the presence of transcriptional inhibitors and marked changes in the pattern of the protein synthesis. This process is known as maternal–embryonic transition (Telford *et al.*, 1990; Lee *et al*., 2014). The maternal-embryonic transition in cattle occurs during the time which the embryo depends on embryonic genome at 8-16 stage. These suggest that the mRNA will be transcript from embryo genome and the pattern of the protein synthesis will change to accommodate the new life of the embryo (Memili & First, 2000).

The maternal to embryonic genome transition is pre-requisite for the activation of a large number of genes and for patterns of expression of genes responsible for successful embryonic differentiation and development (Lequarre *et al.*, 2003; Lonergan *et al.*, 2003a). Furthermore, the pattern of the mRNA of genes from embryo genome regulating embryo development can be useful for molecular markers during the pre-implantation period (Wrenzycki *et al.*, 2004; Rodriguez-Alvarez *et al*., 2010). The mRNA expression pattern of genes regulating embryo development is regarded as one of the relevant parameter determining embryo quality (Wrenzycki *et al.*, 1999; Farin *et al.*, 2004; Dode *et al.*, 2006). Moreover, abnormalities that can results in failure of the embryo development and thus compromise the survivability of the embryo before and after cryopreservation, transfer and implantation can occur through the alteration of the mRNA expression. (Niemann & Wrenzycki, 2000; Yang & Rajamahendran, 2002; Wrenzycki *et al.*, 2004). Analysis of transcript related to embryo quality by means of gene expression technique has become the most powerful tool for determining embryo quality (Warzych *et al*., 2007). In embryo development, the expression of genes such as Connexin 43 (Cx43), Glut-1, the housekeeping X-linked gene glucose-6-phosphate dehydrogenase (G6PD) and HPRT (Hypoxanthine-guanine phosphoribosyltransferase) are amongst those

genes responsible for physiological functions that assist in the development of embryo prior transfer (Table 2.1).

Table 2. 1 List of gene transcript in cattle oocytes and embryos related to their function throughout prei-mplatation development.

Genes	Function	Reference
Cx43(Connexin 43)	Regulate the exchange of small metabolites and ions (up to 1 kDa) between adjacent cells and thereby coordinate metabolic and electrical activities. Gap junctions are thought to play a crucial role in regulating cell growth and development in cattle embryos.	Wrenzycki, *et al*., 1996
Glut-1(glucose transporter-1)	Glucose transporter, Basal glucose uptake; transport across blood-tissue barriers	(Lee, 2001)
Hsp70(heat shock protein 70)	Refolds and maintains denatured proteins *in vitro*	
G6PH(glucose-6-phosphate dehydrogenase)	Candidate for involvement in sex differences, An important role of the enzyme is to detoxify oxygen radicals	(Jolly *et al.*, 2000)
HPRT(Hypoxanthine-guanine phosphoribosyltransferase)		
Insulin growth factors (IGF,IGF-IIR and IGF-1)	Embryonic growth factors with pleiotropic mitogenic activity.	(Wrenzycki, *et al*., 2002a).
OCT-4(octamer-binding transcription factor),	Transcription factor initiating transcription.	(Wrenzycki *et al.,* 2005
BAX	Pro apoptotic regulator	(Wrenzycki *et al.,* 2005
Bcl-xl	Anti-apoptotic regulator, inhibitor of apoptotic proteins.	(Jolly *et al.*, 2000)
polyAtail	MRNA processing	(Wrenzycki, *et al*., 2002b).

Expression patterns of genes has the ability to become markers for embryo quality because it is useful for important biological processes such as compaction, metabolism, transcription and

translation, DNA methylation, growth factor/cytokine signalling, cell cycle regulation during pre-implantation and post implantation stages, (Wrenzycki *et al*., 2004).

Genes in embryo life are expressed differently at different stages and show two expression pattern: the first expression pattern starts at 8-16 cell after embryonic genome activation and the embryo at the time depends completely on embryonic activity. The second starts just before and after embryonic genome activation. At this stage the embryo depend on the maternal and embryo transcriptional pattern (Niemann & Wrenzycki, 2000). Genes expressed during pre-implantation of the embryos are involved in different stages of embryo development and growth (Silva *et al*.,2013). Moreover, characterization of these genes will identified those that are altered during abnormal development. For proper development in embryos, there must be a relationship between the phenotype and gene expression profile this ensures clinical effect of the changes and clarifies the expression pattern of genes necessary for pre-implantation growth (Ponsuksili *et al*., 2002). Therefore, for this reason, identification of pre-implantation genes on cattle embryos would provide information towards the success of embryo production and evaluating the existing protocols to more suitable conditions for pre-implantation and post-implantation developments. In Table 2.2 and 2.3 the different genes involved in different pre-implantation stages on an embryo are illustrated.

Table 2. 2 Illustration of oocyte growth and gene indication genes associated with each developmental stage (Wrenzycki *et al.,* 2005).

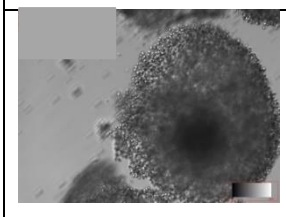
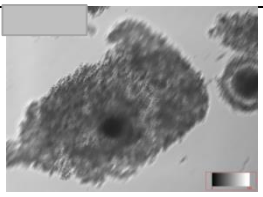
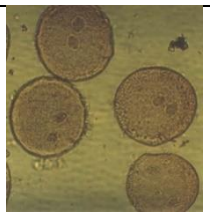
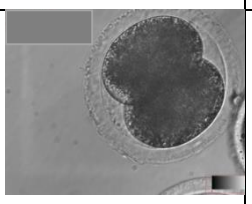
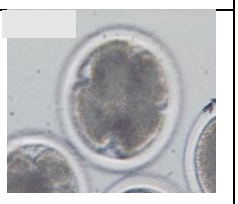
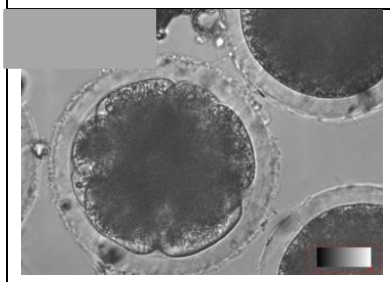
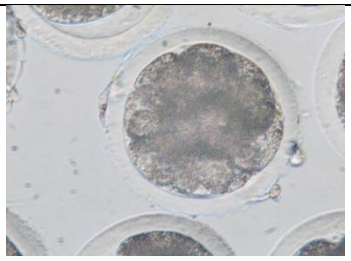
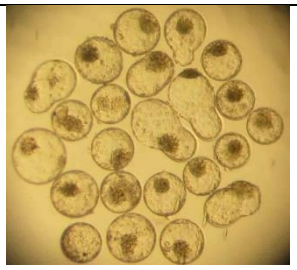
<table>
<tr><td>Oocyte growth</td><td>Oocyte maturation</td><td colspan="3">Time of first cleavage or minor activation of embryonic genome.</td></tr>
<tr><td>Immature oocyte</td><td>Mature oocyte</td><td>zygote</td><td>2 cell</td><td>5-cell</td></tr>
<tr><td>  </td><td>  </td><td>  </td><td>  </td><td>  </td></tr>
<tr><td>polyA</td><td>Cx43,Hsp,G6PH,Oct-4,HPRT and GLUT-1</td><td colspan="3">Zygote:GLUT-1,CX43
2 cell onwards:Oct-4,Hsp and glut-1
4 cell onwards: HPRT, and Hsp,glut-1</td></tr>
<tr><td colspan="2"></td><td colspan="3"></td></tr>
</table>

Table 2. 3 Expressed transcript after the major activation of embryonic genome (Wrenzycki *et al.,* 2005)

Expressed transcript after the major activation of embryonic genome		
8-16 cell	Morula	Blastocyst
		
CX43, GLUT-1, HSP, G6PD and BAX.	CX43, GLUT1, HSP, G6PD, HPRT and BAX.	CX43,GLUT1, G6PD

Different genes are expressed differently in different *in vitro* culture mediums with different supplementations (Silva *et al*., 2013). The connexin 43 gene is not detected in medium supplemented with serum but it is unregulated in the medium supplemented with polyvinyl alcohol. Glucose transporter 1 gene is standard in serum supplemented medium and up regulated in polyvinyl alcohol supplementation medium (Watson *et al.*, 2000). However, heat shock protein is unregulated in serum and standard in polyvinyl alcohol. It is therefore clear that among various IVM media and chemical supplementation, mRNA expression pattern can differ (Watson *et al.*, 2000). The expression of genes in different supplementation mediums do not show differences in gene expression patterns. However, the period of post fertilization culture environment can have a significant impact on mRNA abundance (Lonergan *et al.*, 2003b). Moreover, the survival of an embryo in a specific culture system can be done by monitoring the levels of mRNA and this could an indirect way of quality assessment in the embryo (Liu *et al*., 1997 & Park *et al*.,2006). This further generate information on expression patterns used as genetic markers and gives an indication of abnormal embryo during their pre-implantation stages. In a transcriptome experiment using a bovine-specific microarray, 26 genes were down-regulated in cattle embryos between Day-7 blastocysts and Day-14 conceptus, while more than 500 genes were up-regulated and most of them continued to be expressed until the time of implantation (Rodriguez-Alvarez *et al.*, 2010). Table 2.4**,** summarize the differences in the relative abundance of gene transcripts in cattle embryos grown in culture medium (T-199) with different supplements **(**Wrenzycki *et al*., 1999).

Table 2. 4, Difference in gene transcripts on bovine embryos produced in culture medium (T-199) with different supplements

Transcript	serum	Polyvinyl achol	Embryonic stages
Connexin43	-	↑	8–16 cell. morula., blastocyct.
Glucose transporter-1	+	↑	8–16 cell.
Heat shock protein 70	↑	+	Oocyte maturation–morula.
Poly(A) polymerase	+	↑	Maturation, 8–16 cells.

-, not detected**,+** standard against which the relative abundance of the respective transpcript was compared, ↑ , upregulation.

Genetic factors influencing in vitro embryo production.

Embryo development can be affected by various factors resulting in cleavage and gene transcript to cease (Betts & Madan, 2008). Moreover, in embryo production, most of the embryo block that can affect development occurs mostly between the fourth and fifth cell cycle transition (Memili & First, 2000). The embryonic development block occurs simultaneously with the maternal embryonic transition and embryos that cannot produce their own embryonic genome transcription die due to this block. The developmental block can be caused by two main mechanisms namely: inability to overcome the chromatin repression and activate transcription of important developmental genes and injury reaction caused by environment (Betts & King, 2001).

Effect of glucose in cattle embryo production system

In mammalian cells, glucose is one of the main source of energy with the glucose transporter as primary entrance for uptake of glucose in mammalian cells. Glucose transporters (GLUTs) are glycoproteins encoded by a family of SLC2A (Trehalose inhibits solute carrier 2A) genes. The SLC2A genes are responsible for the uptake of several monosaccharides including glucose, fructose, mannose, galactose and glucosamine (Das, 2012). Transcription of genes such as glucose transport-1 and HSP70 play a fundamental role in the embryo production and when their transcriptions are altered they can cause embryonic cell death. The expression level of genes related with embryo metabolism such as the glucose transporters Glut-1 and

Glut-3 as well as genes from the insulin-like growth factors system (IGF2, and receptors IGF-1r, -2r) also changes during cattle embryo elongation.

Heat stress in bovine in vitro embryos.

Ritossa in 1862 was the first to discover heat shock response after identifying puffs of Drosophila salivary gland chromosomes due to temporary heat shock exposure. During activation of embryonic genome both mouse and cattle embryos become resistant to heat shock exposure, that is 1-2 cell stage in mouse and from 8-16 stage in cattle (Jolly *et al.*, 2000). Exposure of cell to mild heat shock activates thermotolarance and therefore the thermotolarance effect can assist the cell to become more resistance to severe heat shock. These phenomena can be identified in cattle blastocyst but not in earlier stages of embryos (Mortensen *et al*., 2010).

In cattle embryos, the cleavage stage become more susceptible to damage by heat stress (Badr *et al*., 2007). Moderate heat exposure on pre-implantation developmental stages of embryos can cause more damage and cease development. This was observed in Rivera *et al*. (2003) whereby exposure of mouse embryo development to 39˚C at 1 cell was inhibited while in cattle, the developmental embryo was decreased when exposed to 41 ˚C at 4 hours. Embryos after 2-4 stage cell when they approach subsequent development their hypersensitivity towards heat stress become reduced in the culture environment (Silva *et al*., 2013). One of the major cause of mortality in embryo production on different species such as cattle, rabbit and sheep is susceptibility to heat stress (Rivera *et al.,* 2003). However, the cell can be able to act against the elevated temperatures through the cell- protective processes such as the cytoprotection or thermotolarance. Moreover, the cell can induce the expression of HSP genes that protect the cell from thermal challenges in further developmental processes of the cell. There's differences in production between *in vitro* and *in vivo* embryos. The *in vitro* embryo can increase lipid content in the cell, exhibit retarded development rate or lower the survivability after freezing (Hansen, 2009).

In animal reproduction, *in vitro* embryo production is considered as one of the most suitable model to study the mechanism influence of high temperature. These is because of the production processes of producing embryos *in vitro*, such as oocyte maturation, fertilization and subsequent embryo development (Duszewska *et al.,* 2010) which allows the examination of the processes related to thermosensitivity and thermoresistance of gametes and embryos (Zeron *et al*., 2001; García-Isperito *et al.,* 2007). Increasing the temperature of embryo culture

from 40-41°C in most *in vitro* experiments is used to induced stress and most of the control temperature used ranges between 38 and 39°C (Rynkowska *et al.,* 2011).

Roth & Hansen (2005), demonstrated that, the disruption in oocytes maturation after thermal stress is due to the changes in karyokinesis. Most of the oocytes exposed to high temperature (41°C) are arrested at the first meiotic metaphase. This may be due to the incorrect chromosomal segregation during anaphase caused by the activation of meiotic spindle check point when oocytes are exposed to elevated temperatures (Brunet *et al.,* 2003). Meiotic spindle check point is activated when incorrect arrangement of chromosomes in metaphase plate or incorrect attachment of chromosome to the spindle is recognized, and consequently leads to anaphase delay (Zhou *et al.,* 2002; Cleveland *et al*., 2003). Roth & Hansen (2005), reported that heat stress in cattle oocytes also shows deformation of meiotic spindle, a decrease in the amount of actin connected to the membrane, and increase in amount of actin dispersed in the cytoplasm. The sensitivity of heat stress differs in different stages of embryo production. Oocytes, morula and blastocyst are less sensitive to heat stress compared to embryos at 2-4 cell stage (Rivera *et al.,* 2004a). In 2-4 cell cattle embryo stage, they might be the production of free radicles due to the activity of the mitochondria which makes the embryos highly thermosensitive as a results of thermal stress (Hansen, 2007). According to Rivera *et al.*(2004b), the changes that occurs inside the blastomere that are considered to be impossible to overcome by genome activation at the 8 cell stage can also inhibit embryonic development by elevated temperature.

Elevated temperatures do not activate apoptotic signals in 2-4 cell stage and the first apoptotic cells do not appear until 8-16 cell stage in cattle and not earlier than 4 days post fertilization (Matwee *et al.,* 2000; Paula-Lopes & Hansen, 2002). According to Rynkowska *et al.* (2011), the above suggest that, activation of apoptosis depends on the number of mitotic division and the time past fertilization in pre-implantation of cattle embryos. This suggests that those embryos that have passed through the activation of embryonic genome are more resistant to elevated temperature because during these time embryos are already able to induce programmed cell death and it is possible to eliminate damaged cells from the embryo (Rynkowska *et al.,* 2011).

One of the mechanisms that the oocytes and embryos protect themselves against damages from external factors including heat shock id through the production of heat shock protein 70. The HSP 70 is an important element of thermoresistance in cattle gametes and function both in the cytoplasm and the nucleus. These proteins can recognise incorrect folding of other proteins and assist in them to fold correctly (Rynkowska *et al.,* 2011). They can also assist

newly synthesized proteins during their folding. In other cases, they can newly synthesis other proteins and select dysfunctional proteins and direct their discharge to their degradation site (Rynkowska *et al.,* 2011). Moreover, HSP 70 protects cells from heat shock by stabilizing ribosomal RNA (Duncan & Hershey, 1989; Nover & Scharf, 1991). Hendrey & Kola (1991), concluded that for successful embryonic production oocytes became more resistant to heat shock when microinjected with HSP 70.

2.4. **Cell apoptosis and Bcl-2 protein family genes**

Apoptosis has been first discovered by Kerr *et al*. (1972) by identifying electron morphological analysis of a dying cell. It is a process dependent on morphologically and biochemically stimuli (Yang & Rajamahendran, 2002). During apoptosis a condensed and misshapen nuclei with clumped chromatin smaller than intact healthy nuclei can be observed. A cell that shows signs of apoptosis tend to rounds up and separate from its neighbouring cells (Opiela, 2009).

Apoptosis in cattle consists of two pathways, the intrinsic apoptotic pathway that involves the mitochondria and an extrinsic apoptotic pathway that involves death receptors (Cleary *et al*., 1986; Melka *et al*., 2010). The mitochondrial pathway of apoptosis in mammals is regulated by members of the B cell lymphoma-2 (BCL-2) family of proteins (Melka *et al*., 2010). Bcl-2 family proteins are key regulators of the apoptotic process. The Bcl-2 proto oncogene was originally identified from a human chromosomal translocation that predisposed affected individuals to malignant transformation of immune cells (Cleary *et al*., 1986).

In apoptosis the Bcl-2 related genes are known to be regulatory genes in the apoptotic pathway (Hossini *et al.,* 2005), this gene family may either suppress or promote the presence of apoptosis in mammalian embryos (Muscarella *et al.,* 1998). In the mitochondria, the Bcl-2 proteins are the key regulators of the permeability of mitochondrial membrane. These proteins are divided into two groups: the pro-apoptotic which promotes apoptosis (Bcl-XS, Bid, Bax, Bak, Bik and Bad) and anti-apoptotic proteins (Bcl-W, Bcl-XL, Bcl-2, A1 and Mcl-1) that works to prevent cytochrome *C* release to initiate apoptosis (Shamas-Din *et al*., 2013). In table 2.4 shows the locations of the bcl-2 family genes are shown.

Table 2.5 Subcellular localization of BCL-2 family members as provided in genebank (Youle & Strasser, *2008*). http://www.ncbi.nlm.nih.gov/gene/ and http://www.ncbi.nlm.nih.gov/unists).

BCL-2 family member	Chromosome no	ALIASES	Location in healthy cells	Location in apoptotic cells
Anti-apoptotic members				
BCL-2	Chromosome 5, AC_000162.1 (98196412..98233551, complement)	BOS_5853	Mitochondria and ER	Mitochondria and ER
BCL-XL	Chromosome13, AC_000170.1 (61766800..61817390, complement)	BOS_13290, BCLX, Bcl-xL	Cytosol, mitochondria and ER	Mitochondria and ER
BCL-W	Chromosome 23, AC_000180.1 (7654652..7661915, complement)	BOS_21299	Cytosol and mitochondria	Mitochondria
MCL1	Chromosome 3, AC_000160.1 (20172164..20176968)	BOS_2960	Cytosol, mitochondria and ER	Mitochondria
Pro-apoptotic members				
BAX	Chromosome 18, AC_000175.1 (55985166..55989379)	BOS_17596	Mainly cytosolic, although in cultured cells, up to 50% of the BAX can attach lightly to the mitochondria	Mitochondria and ER
BOK	Chromosome 5, AC_000162.1 (114542464..114571825)	BOS_6166	Cytosol and mitochondria	Mitochondria
BID	Chromosome 29, AC_000186.1 (43190195..43202349, complement)	BOS_24844	Cytosol	Cytosol and mitochondria

At the time of stress or damage in the cell, the pro-apoptotic members of the Bcl-2 family proteins such as Bax or Bak will be activated at the mitochondria. Activation of these proteins results in the formation of pores outside the mitochondrial membrane which then lead to the escape of pro-apoptotic molecules, including caspase and cytochrome *C*. The release of mitochondria-derived activator cytochrome *C* will then activate the release of members of the

proteases called the caspases that cleave the key cellular proteins to initiate death (Brunelle & Letai, 2009).

The Bcl-2 family member genes in cattle pre-implantation embryo

Apoptosis can be primarily identified in cattle embryo morphology. During the pre-implantation stage of the embryo, a number of stimulus can cause the cell to enter its apoptotic phase through the interference of the balance between the pro- and anti-apoptotic proteins which identifies either susceptibility or resistance to apoptosis (Johansson *et al*., 2010). Once the embryo cell enters its apoptotic phase, the cascade of caspases is activated and it cleaves the specific downstream target and cause a number of irreversible damages such as organelle dysfunction, membrane blebbing, cytoplasm shrinkage, cellular degradation, formation of apoptotic bodies and condensation of nuclear chromatin (Jolly *et al.*, 2000).

During oocyte and embryo development, different members of the Bcl-2 protein family are expressed differently (Boumela *et al*., 2009). Amongst the pro-apoptotic members, the Bax is the most dominant member in oocytes and embryo under stressful conditions. Furthermore, Melka *et al*. (2010), reported that the expression of the Bax to the oocytes and embryo in cattle suggest that the pre-implantation development may be under permanent threat of death. These will then mean that the survival of the embryo will depend on the inhibition of its pro-apoptotic activity and the release of the anti-apoptotic member the Bcl-2 gene. Furthermore, the high expression levels of Bcl-2 expression suggest that the embryo may have a chance to supress the expression of Bax activity (Boumela *et al.*, 2011).

The apoptosis pathway in mammalian cells including cattle embryos start at the activation of the pro-apoptotic members of the bcl-2 family such as Bax, Bid or Bad to the mitochondria. However, each pro-apoptotic member works at specific settings for the initiating apoptosis (Wilfling *et al*., 2012). For example, Zimmermann *et al*. (2001), revealed that Bax is responsible in DNA fragmentation apoptosis, Bid initiate Fas-mediated apoptosis and Bad is activated in neurotrophin deprivation-induced death. The activation of these pro-apoptotic proteins will release the cytochrome *C* protein contained within the intermembrane space of the mitochondrion (Wilfling *et al*., 2012) The cytochrome *C* in the mitochondria will then be couples with a heme group to became holocytochrome *c*. It is in this form that the cytochrome *C* can induces caspase activity (Yang *et al*., 1997). The caspases are a family of the cysteine proteases and they initiate the phases of apoptosis (Brad *et al.,* 2007). Apoptosis is initiated by caspases through cleavage DNAse 1 enzyme within the caspase and this enzyme

mediates biochemical and mophologcal changes such as DNA fragmentation and plasm membrane blebbing (Ko¨hler, 2002).

Once the caspase is released through the formation of apoptosome which is the combination of cytochrome c, apoptosis protease activating factor-1(Apaf-1) and procaspase 9, the cell will officially undergo apoptosis (Zimmermann *et al.*, 2001). Apaf-1 is an apoptotic protein that reside within the cytosol in the mitochondria and when it binds to the cytochrome *C* it induces it to oligomerize. It consequently activates Pro-caspase 9, which then causes the apoptosome to recruit pro-caspase-3. Pro-caspase 3 will then be released to mediate apoptosis as shown in figure 2.2.

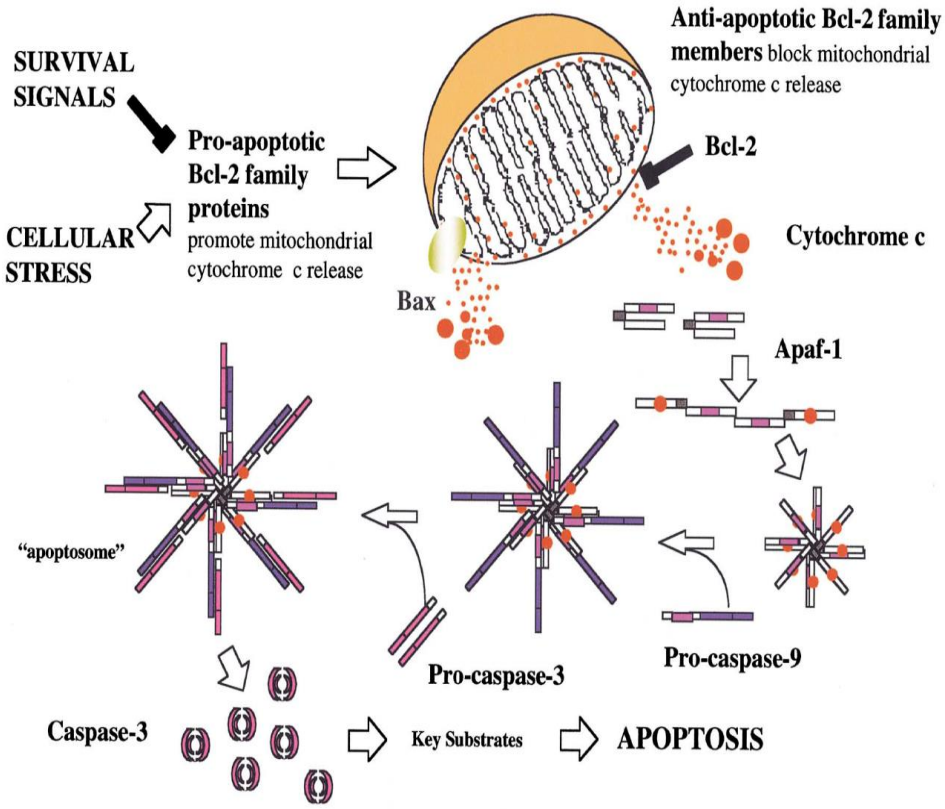


Figure 2. 2 . Induction of apoptosis in the mammalian cell via the mitochondrial pathway (Zimmermann *et al*., 2001)

According to Zaraza *et al.* (2009), apoptosis in cattle embryos is observed when embryo genome is activated at 8 cell stage of development. Poor development of embryos in cattle as a result of external stimulus is often associated with apoptosis and this lead to the death of the embryo prior compaction (Zaraza *et al.,* 2009). However, the is no discovery of apoptosis incidences either morphologically, biochemically or molecular in cattle embryo prior the 8 cell stage (Byrne *et al.,* 1999; Matwee *et al.,* 2000; Gjørret *et al.,* 2003, Betts & Madan 2008). Conversely, several reporters (Matwee *et al.,* 2000; Brad *et al.,* 2007& Gjørret *et al.,* 2007) emphasised that even though the 2-4 cell stage embryos can be under apoptosis attack due

to mitochondrial depolarization or inhibition of protein kinases, apoptosis will be partially activated and the embryo will exhibit some caspase activity and have limited DNA fragmentation. The above suggest that embryos at 2-4 cell can have all the apoptotic machinery due to stimulus however they are not affect mainly due to the immature mitochondria and they might be non-ascertained factors that assist them to overcome some inhibition of apoptosis pathway (Weil *et al.,* 1996; Brad *et al.,* 2007; Bakri *et al*., 2016).

Factors influencing apoptosis in cattle embryos.

The stimulus for apoptosis in cattle embryos can be within the embryo cell such as excess production of oxidative species and DNA damage or extracellular such heat stress (Vandaele *et al*., 2008). The damage that is due to DNA fragmentation on the embryo cell can be shown by excess increase in the expression of the transcription factor, the tumour protein 53 (TP53) to most pro-apoptotic gene (Betts & King 2001). Moreover, according to Yu (2015), the caspase-associated DNase (CAD) which is a magnesium-dependent endonuclease specific for cutting dsDNA to generate 3-hydroxy ends, is a bench mark of apoptosis. The Bcl-2 expression, however, during the growth of an embryo specifically at the blastocyst stage appears to be restricted to specific cells of the inner mass, whereas Bax has been shown to be localized in the cytoplasm of the blastomeres which are the trophectoderm cells and the inner cell mass (Viuff *et al.,* 2001; Vandaele *et al*., 2008).

The artificial environment of the *in vitro* embryo production processes such as IVM, IVF and IVC can influence apoptosis. During the *in vitro* processes, cellular stresses, imbalance of nutrients and chromosomal abnormalities can occur and these therefore activate the presence of apoptosis to the growing embryos and affects its ability to reach the blastocyst stage (Viuff *et al.,* 2001 & Knijn, 2004). The activation of embryo genome plays a vital role in apoptosis activation because improper activation of these process can trigger the release of apoptosis factors in cattle. This is evidence of the detection of apoptosis from the 6-8 cell stages in cattle and less detection at the earlier stage before embryo genome activation (Byrne *et al.,* 1999; Gjørret *et al.,* 2003). During the summer season the higher outside temperature affect embryo production processes (oocyte maturation, fertilization and embryo development) *in vivo* embryo production which can leads to apoptosis (Rynkowska *et al.*, 2011). Moreover, apart from all inducers of apoptosis, heat stress is one of the main factor that triggers embryo apoptosis (Hansen, 2009). It is therefore suggested that for the success of the *in vitro* embryo production, a balance of pro and anti-apoptotic proteins is important (Matwee et al.,2000; Yang & Rajamahendran, 2002)

In the study by Roth & Hansen (2005) it was emphasized that in all the process of embryo production, it is the nuclear maturation of oocytes that is most affected by high temperature and results in disruption of the nuclear. Dorado *et al.* (2001), consider that it affects mainly cytoplasmic maturation. high temperature affects the joining of the maternal and the paternal and therefore delay the first mitotic division of the zygote because of the disruption of the cytoskeleton (Rivera & Hansen, 2001). In the case of high temperature, it is not only the division of the zygote that is affected but also the potential of the zygote to develop further and become an embryo. Moreover, the affected embryo can have suppression of the expression of fundamental gene abnormalities in the embryo and cell death (Rivera & Hansen, 2001).

The apoptotic cells in mammalians can be seen by the presence of fragmentated DNA, shrinkage of the cell and segmentation of the cell into apoptotic bodies (Opiela, 2009). DNA fragmentation can have severe cellular consequences and it is divided into two categories namely the lesion and strand breaks. The lesion DNA fragmentation damage can cease the transcription and replication processes in the DNA, these therefore results in the production of mutation due to changes in the chemical and physical structure of the DNA (Doherty & Jackson, 2001; Sturmey *et al*., 2009). The lesion damage can be formed by stimulus such as ROS. The strand breaks damage of the DNA fragmentation can either be a single strand breaks (SSB) which can still activate cell death or in worst cases can be a double strand breaks (DSB) (Doherty & Jackson, 2001).

Methods used in assessing apoptosis in bovine embryos

There are number of techniques for apoptosis detection in cattle embryos both morphologically and genetically. One of the morphological technique that is used in embryos is the TUNEL method. The TUNEL method uses the deoxynucleotidyl transferase (TdT) enzyme. These enzyme allows the evaluation of DNA breaks by binding to the 3-hydroxyl end of the DNA breaks. Moreover, the breaks can be highlighted by fluoresce of the TdT enzyme through fluorescent microscopy (Vandaele *et al*., 2008; Yu, 2015). The molecular evaluation of apoptosis in embryos can be achieve through the use of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). RT-PCR is used by analysing the relative transcript in embryos. It incorporates the use of housekeeping genes such as the rabbit-globin RNA prior extraction of the embryo samples as a standard for estimation the quantity of the RT-PCR product (Opiela, 2009). With the use of RT PCR procedures, it is possible to gain insight into the transcriptional activity of individual oocytes and embryos by determining the relative abundances of transcripts from various developmental important genes (Wrenzycki, 2000).

Caspase activity in cattle embryos hold a central position in apoptosis. It cleaves the caspase-activated DNAse substrate that is responsible for the DNA fragmentation through breaking of the ends of the DNA (Sakahira *et al*., 1998). Therefore, apoptosis in cattle embryos can also be determined by measuring the activity of the caspase enzyme. The enzyme can be detected microscopical by the use of fluoroprobe. flouroprobe is used to incubate the embryos for an hour and the caspase activity post incubation will be assessed by fluorescence microscope (Paula-Lopes & Hansen, 2002).

2.5. **Conclusion**

In vitro embryo production provides an excellent model of the assisted reproduction technology that contributes to increase number of offspring in beef cattle. However, the artificial environment can have detrimental effects on the embryo development. The artificial environment in IVEP may result in apoptosis due to the alteration of gene expression transcripts. Moreover, evaluation of apoptosis may serve as molecular markers for embryonic quality and identification of embryonic abnormality.

Chapter 3

Materials and methods

3.1. Introduction

The research project focuses on factors involved in apoptosis on *in vitro* produced embryos. Detection of apoptosis methods such as d-UTP nick end-labeling (TUNEL), which allows the analysis of apoptosis in fragmented mammalian embryonic nuclei cells and caspase-3 activities which initiate apoptosis in the mitochondrion by the release of DNAse enzymes are of key importance for the evaluation of apoptosis on *in vitro* beef cattle embryos in these project. This chapter discuss the procedures used to achieve the three objectives done in this study. Briefly it involves beef cattle ovary collection, retrieval of oocytes to produce embryos *in vitro*, assessment of cell apoptosis in beef cattle *in vitro* embryos.

The study was approved by the Ethical Animal Care committee of the University of Pretoria (EC 140526-051) and the Ethical committee of the Agricultural research council (API).

3.2. Material

3.2.1. Research facilities

The experiments were conducted at the Agricultural Research Council: Germplasm Conservation and Reproductive Biotechnologies (GCRB) laboratories (25˚ 55’ S; 28˚ 12’ E) in Pretoria (Irene) South Africa.

3.2.2. Material

The study consists of four experiments. Samples from all the experiments were obtained from *in vitro* produced Bos-indicus beef cattle oocytes and embryos. Cattle ovaries were collected from Morgan abattoir three times a week, Monday, Wednesday and Fridays after slaughter. Maximum of 30 cow ovaries were collected within one hour after slaughter and transported in a thermo flask containing buffer saline (Sigma Aldrich®, Germany) at a temperature of 38°C. Upon arrival at the laboratory, oocytes were immediately retrieved by aspiration method as described under point 3.2.1(figure 2). A total of ± 900 oocytes were collected per week for the study over five-month period. An amount of ±1200 oocytes were used for maturation experiments from the study and a total of ±16800 were used for the embryo production studies.

3.3. **Methodology**

Table 3.1 provides a brief summary of the four experiment conducted in this study.

Table 3. 1. Summary of primary activities performed for the four experiments

Experiment	Activity
Experiment 1	*In vitro* maturation at four temperature (39 , 41, 42 & 43 °C) and subsequent embryo production post maturation using two favourable maturation temperature.
Experiment 2	Hoechst 33323 staining of produced blastocyst.
Experiment 3	Caspase 3 activity on *in vitro* embryos using 96 well thermoscientific microplate reader (BioTek, USA).
Experiment 4	Assessment of DNA fragmentation on *in vitro* embryos using tunel assay method recommended by Opiela (2009).

The first experiment was aimed at investigating the extrusion of the first polar bodies post *in vitro* oocyte maturation at four different temperatures namely: 39, 41, 42 and 43 °C. The extrusion of the first polar bodies were examined 24hours post maturation. Oocytes matured from the most favourable two maturation temperatures (39 and 41 °C) were used for subsequent embryo development. For subsequent embryo production, all oocytes from both groups (39 and 41 °C) were subjected to the normal embryo production (39 °C, 5 % Oxygen, 5 % Carbon dioxide and 100 % humidity (Rynkowska *et al.,* 2011).

The second, third and fourth experiments were performed from embryo samples that were selected from oocytes matured at 39 and 41 °C incubation temperatures. Embryos were divided into groups namely embryos before embryonic genome activation (2-4 cell stages), after embryonic genome activation (≥ 8 cell stages) and Blastocysts (Memili & First, 2000). A total number of 200 embryos were used for each experiment. From the 200 embryos, 100

were for oocytes matured at 41 °C and 100 for oocytes matured at 39 °C group. Each experiment was repeated six times.

The second experiment aimed at morphological evaluation of produced embryos from both (39 and 41 °C) matured oocytes. At day 7 of embryo production, blastocyst from oocytes on both maturation temperature groups were stained with Hoechst 33323 (Sigma Aldrich®, Isreal) for nuclei cell count.

The third experiment investigated the caspase 3 activities on *in vitro* produced embryo following 39 and 41°C maturation temperatures, while the fourth experiment involved evaluation of fragmented DNA of *in vitro* embryos matured at 39 and 41 °C incubation temperatures using tunel assay method (Opiela, 2009).

3.4. ***In vitro*** **embryo production**

3.4.1. ***In vitro*** **oocytes maturation**

Prior to retrieval of oocytes, the ovaries as represented on figure 3.1 were washed twice with buffer saline (Sigma Aldrich®, Germany) to remove blood contamination from slaughtered donor cows and they were sprayed with 70% alcohol (Ethanol, Sigma Aldrich®, Germany) for further preclusion of contamination. The oocytes were then retrieved by aspiration method shown in figure 3.2 that required 18-gauge needle and 10ml syringe (Bohlooli *et al*., 2015).

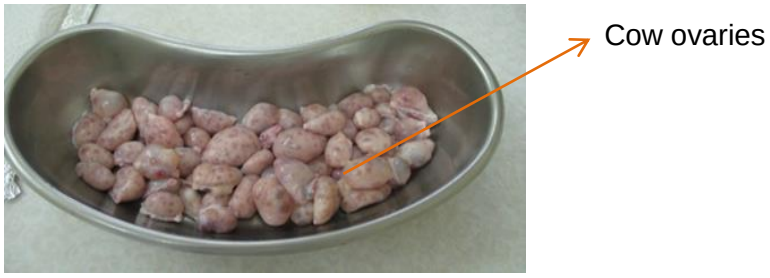


Figure 3.1 Beef cattle ovaries (ARC- Germplasm Reproduction Biotechnologies (GCRB) lab).

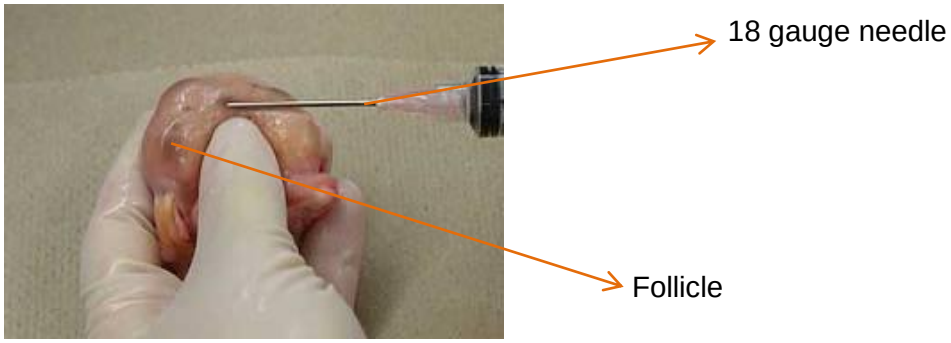


Figure 3. 2 Aspiration method of oocytes retrieval (ARC- GCRB lab)

Post oocytes retrieval, cumulus oocyte complexes (COCs) with greater than or equal to three layers of cumulus cells (Melka *et al*., 2010) were selected under Olympus microscope (New York microscope Co, USA) as represented in figure 3.3. The selected COCs were washed twice, firstly in 3 ml tissue culture medium (M-199 + 10% FBS (Fetal bovine serum (Sigma Aldrich®, Germany) three times and secondly washed in 3 ml modified Dulbecco phosphate buffer saline (DPBS, Sigma Aldrich®, USA) three times. The COCs post washing were randomly allocated into 39, 41, 42 and 43 °C maturation temperatures and matured in 500 µl of M-199 + FSH/LH/EST (40 µl Follicular stimulating hormone, Sigma Aldrich®, USA)/20 µl LH (Luteinizing hormone, Sigma Aldrich®, USA) and 20 µl EST (Estradiol, Sigma Aldrich®, USA) maturation medium covered with 250 µl of mineral oil (Sigma Aldrich®, USA) at 5 % CO_2, 5 % O_2 and 100% humidified air then incubated for 24 hours for *in vitro* maturation. The oocytes were 50 per maturation well.

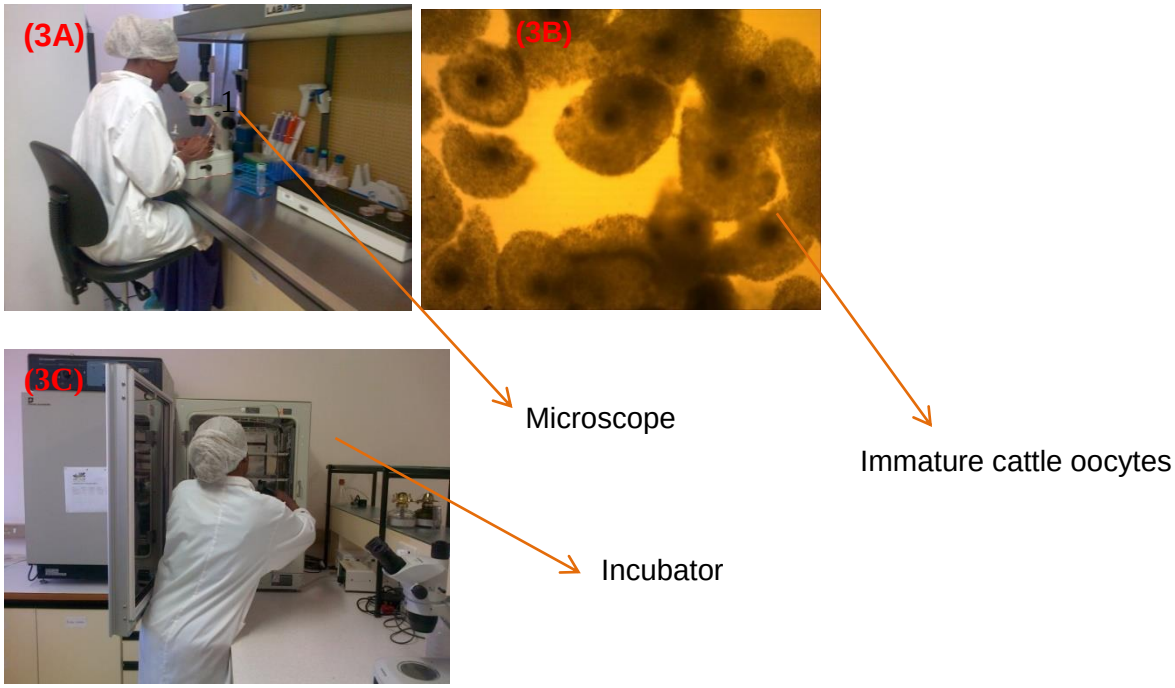


Figure 3. 3 Bovine oocytes *in vitro* maturation performed at the ARC lab, GCRB section**.**
3 A= microscope for oocyte search, 3 B= oocytes and 3 C= incubator

3.4.2**. Polar body extrusion**

After 24 hours of maturation, oocytes from each temperature were removed from the incubator and vortexed in 200 µl of M-199 medium supplemented with FBS (Sigma Aldrich®, Germany) (M-199 + 10 % FBS, Sigma Aldrich®, Germany) for 1 minute 30 seconds and were transferred into a dish containing 3 ml of (M-199 + 10 % FBS, Sigma Aldrich®, Germany) for polar body extrusion. The assessment was performed using Olympus 1x 71 microscope (New York microscope Co, USA) at 20x/0.45 Rc_2 magnification. Based on the polar body extrusion

outcomes from the four incubation temperature, the preferred two incubation temperatures were selected for subsequent embryo development.

3.5. ***In vitro*** **fertilization**

Matured oocytes preparation for fertilization

Matured oocytes were washed five times in five drops of 100 µl pre-warmed Bracket and Oliphant (BIO-IVF) medium covered with 250 µl of mineral oil (Sigma Aldrich®, Germany). The BIO-IVF medium consists of: Sodium chloride (NaCl, Sigma Aldrich®, USA), Potassium chloride (KCl, Sigma Aldrich®, USA), Sodium phosphate (NaH2PO4.H2O, Sigma Aldrich®, USA), Magnasium chlroride (MgCl2.6H2O Sigma Aldrich®, USA), Calcium chloride (CaCl2.2H2O, Sigma Aldrich®, USA), Glucose (Sigma Aldrich®, USA), Antibiotic-Antimycotic (Gibco, Germany), Phenol Red, NaHCO3 (sodium bicarbonate, Sigma Aldrich®, USA), and Sodium pyruvate (Na-Pyruvate, Sigma Aldrich®,USA). After washing, 20-25 oocytes were then transferred in each of the seven drops of 50 µl of pre-warmed Bio-IVF medium covered with 250 µl of mineral oil for fertilization (Sigma Aldrich®, Germany).

3.5.2. **Semen thawing**

Frozen-thawed Nguni semen straws from a single bull were used for *in vitro* fertilization. Briefly, frozen semen with straw size 0.25 ml (Embryo plus, South Africa) was thawed for 30 seconds in air and a minute in warm water at a temperature of 37 ˚C. Semen was washed twice by centrifugation at 1500 RPM for eight minutes per centrifugation at 37 ˚C in 4 lm of pre warmed sperm wash medium which consist of BIO-IVF solution, Caffeine (Sigma Aldrich®, USA) and BSA (Sigma Aldrich®, USA) based on protocol by Yang & Rajamahendran (2002). After the first centrifugation, the supernatant was discarded and new 4 ml of sperm wash medium was added into the semen and centrifuged for the second time. The supernatant was again discarded for the second centrifugation and spermatozoa pellet remained. Then, 50 µl sperm wash X number of oocytes drops was added in the sperm pellet. The matured oocytes in each drop was then fertilized with 50 µl sperm pellet at a concentration of 265 x 10^6 sperm cells/ml and incubated in Bio-IVF medium for six hours on day 0 of *in vitro* embryo production.

3.6. *In vitro* culture.

Zygotes preparation for in vitro culture

Six hours post insemination presumptive zygotes were removed from fertilization drops and cumulus cells were removed by vortexing in 200 µl of pre-warmed (M199+10% FBS) medium for one minute 30 seconds.

3.6.2. *In vitro* culture of Zygotes

Zygotes were washed five times in five drops of 100 µl pre-warmed synthetic oviductal fluid medium supplement with bovine serum albumen (SOF-BSA) and 20-25 zygotes were transferred in seven drops of 50 µl (SOF-BSA) covered with 250 µl mineral oil (Sigma Aldrich®, Germany) and were then cultured for 48 hours in a modular chamber with 5 % oxygen and 5 % CO_2 mixed gas added for a minute. The SOF-BSA medium consists of Sodium Chloride(NaCl, Sigma Aldrich®, USA), Potassium Cloride (KCl, Sigma Aldrich®, USA), Potassium phosphate monobasic (KH2PO4, Sigma Aldrich®, USA), Magnesium sulfate heptehydrate (MgSO4.7H2O, Sigma Aldrich®, USA), D-Lactic acid sodium salt (C3H5O3, Sigma Aldrich®, USA), Antibiotic-Antimyotic, Sodium bicarbonate (NaHCO3, Sigma Aldrich®, USA), Phenol Red (Sigma Aldrich®, USA), Ultrapure water (CRITI CARE (pty), South Africa), BSA (Sigma Aldrich®, USA) and Pyruvic acid (Sigma Aldrich®, USA). The sperm exposed oocytes were examined for initial cleavage after 48 hours of culture (Day two of *in vitro* embryo production). The embryos were transferred to a new pre-warmed medium of synthetic oviductal fluid supplemented with fetal bovine serum (SOF-FBS) which consist of SOF stock solution and FBS (Sigma Aldrich®, USA) for subsequent embryo development post first 48 hours of culture. At 72 hours of incubation (Day five of *in vitro* embryo production), 20 µl of old (SOF-FBS) culture medium was removed and replaced with 20 µl fresh medium of (SOF-FBS) per culture drop. Seven days after culture blastocyst development was then examined (B r y ł a & T r z c i ń s k a, 2011).

3.6.3. Nuclei cell analysis

Blastocysts developed from matured oocytes incubated at both 39 and 41 ˚C temperatures were removed. Expanded and hatched blastocyst were then washed twice in 3 ml of pre-warmed MPBS medium. 250 µl of Hoechst 33342 (Sigma Aldrich®, Isreal) was diluted in 2.5 ml sterile water (CRITI CARE (pty), South Africa) to make-up stock 1 of the Hoechst solution and store at 4 ˚C. Stock 2 of the Hoechst solution was prepared by adding 8 ml Dulbecco phosphate buffer saline, Sigma®, Germany (DPBS) and 2 ml Glycerol inside a 10 ml tube. On the day of use, 20 µl of Stock 1 was added into 10 ml of stock 2. Based on the procedure of

Bohlooli *et al*. (2015), the embryos were transferred to a clean microscope glasslide (Labcon, Germany). consequently, four small drops of a pen pointer of Vaseline (Johnsons and Johnsons (pty) South Africa) around the embryo drop were made and a cover slip (Labcon, Germany) was placed over the slide and gently squeezed until it touches the embryo drop. A minimum volume of 5 µl of Stock 2 solution were added gently on the sides of the cover slip until the whole area under the slip was covered. The end of the cover slip was sealed using colorless nail polish (Essence, New York), and allowed to dry for three hours before counting the nuclei cells. Staining and mounting of the embryos was performed with the laboratory lights dimmed (Melka *et al*., 2010). Nuclei cell count were evaluated using Computer sperm analyser® v 3.4.0-english version and Olympus B X51 epifluorescence microscope at 60 X magnification, represented on figure 3.4 at the ARC (germplasm lab).

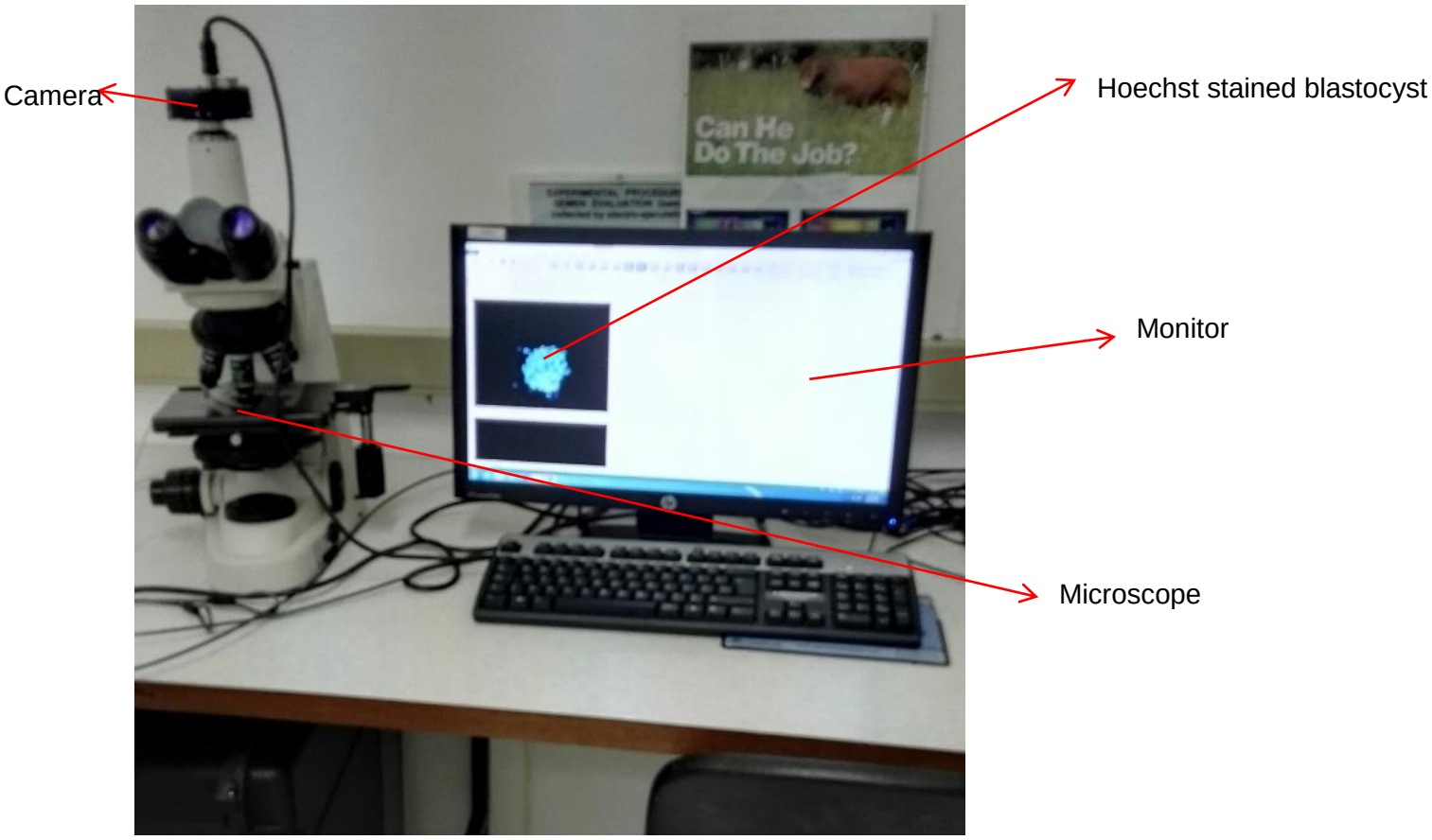


Figure 3. 4 Hoechest blastocyst staining using Computer Sperm Analyser® (Barcelona,Spain) GCRB lab

3.7. **Caspase-3 activity of produced embryos from oocytes matured at two (39 and 41 ˚C).**

A total number of 200 embryos of 2-4 cell development from the oocytes incubated at 41 and the 39 ˚C maturation temperature were removed from the embryo culture medium (SOF-BSA)

at day two of insemination and a total number of 200 embryos (≥ 8 cell) stage were removed at day five, while blastocysts were removed at day seven from the culture medium. All produced embryos were washed three times in 50 µl drops of pre-warmed PBS-PVP. Positive control samples were first incubated for two hours in 50 µl drops of H_2O_2 prior the caspase procedure. Caspase-3 activity assay was performed using a Caspase-3 Colorimetric Activity Assay Kit (EMD Millipore, USA) according to the manufacturer's instructions. The enzyme reactions were then colorimetrically monitored at 405 nm and 450 nm reference filter with a 96 microtiter thermosceintific plate reader (USA) represented at figure 3.5 (Power Wave XS; BioTek, USA) by reading the OD (Optimal density) values (Hwang *et al*., 2012).

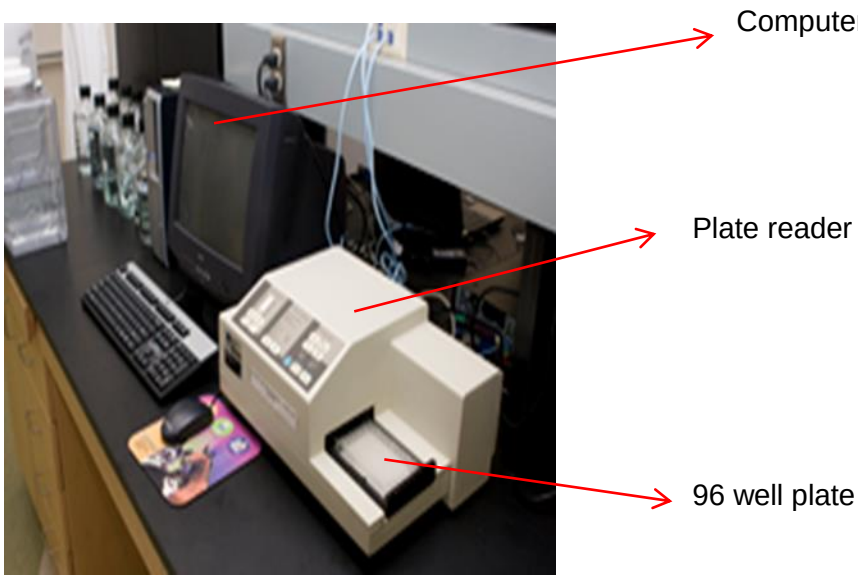


Figure 3. 5 Microplate reader ARC-Nutrition section lab

38. DNA fragmentation of produced embryos from oocytes matured at two (39 and 41 ˚C).

DNA fragmentation was examined using the Tunel assay method (Sakatani *et al*., 2013). Tunel assay was performed using the Alexa Fluor® 488 assay kit (Eugen, USA) according to the manufacture instructions. A total number of 200 embryos of 2-4 cell developed from oocytes incubated at 39 and 41 ˚C maturation temperatures were removed from embryo culture medium SOF-BSA at day two of insemination and a total number of 200 embryos ≥ 8 cell embryos were removed at day five of insemination. Blastocysts were removed on day seven of *in vitro* culture for the tunel assay experiment. Embryos from both development group and blastocysts were washed 3 times in 50 µl drops of pre-warmed PBS- PVP (phosphate buffer saline-polyvinylpyrrolidone, Sigma Aldrich®, Germany) for two min per wash by transferring the embryos from drop to drop. The embryos were then fixed in 50 µl drops of 4 % paraformaldehyde solution (Sigma Aldrich®, Germany) prepared in PBS at pH 7.4 for one h

at room temperature. After fixation embryos were then washed 3 times in 50 µl drop of PBS/ PVP by transferring them from drop to drop (2 minutes for each wash) and Incubated in a 50 µl drop of 0.5 % (v/v) Triton X-100, 0.1 % (w/v) and sodium citrate (Sigma Aldrich®, USA) solution for permeabilization. Post permealization embryos were stored for 30 minutes at room temperature in a humidified box (Silva *et al*., 2013).

Preparing a Positive Control

Positive control embryos were incubated for two hours in 2 % hydrogen peroxide (H_2O_2). Followed by the addition of 100 µl of the DNase I solution mixture to each embryo sample and incubate for 30 minutes at room temperature. The slides were washed once with deionized water (lifetechnologies®, South Africa) and proceed to TdT (Terminal Deoxynucleade tansferase Reaction) Reaction. DNase I solution consists of 89 µl Deionized water, 10 µl DNase I buffer and 1 µl DNase I. Negative control embryos were performed without the addition of DNase I solution mixture. Post negative and positive control preparation, the samples preceded to the fixation and permibilization procedures mentioned in 3.7.

All experimental samples proceeded to the TdT reaction according to the manufacturer instruction using the Click-iT® tunel assay (Eugen, USA). Post completion of the preparation of the samples, the samples were mounted on a slide and covered with a cover slip in a similar procedure performed in 3.6.3 for imaging analysis. Imaging analysis was performed using the Alexa Fluor® 488 emission (Barcelona, Spain) at 519 (nm) (Silva *et al.,* 2013).

TUNEL index was also performed on produced blastocysts from both maturation group to evaluate the percentage of tunel positive. TUNEL index = (no. TUNEL-positive nuclei, fragmented and condensed)/ (total no. of nuclei) × 100 (Bryla & Trzcińska, 2011).

3.9. **Statistical analysis**

Data was collected for polar body status, embryo production, caspase 3 activity and DNA fragmentation on two different temperatures (39 and 41 ˚C). The data was then analysed as a Complete Randomized Design with two treatments replicated six times. An Analysis of Variance (ANOVA) was performed. Shapiro-Wilks test was used on the standardized residuals to test for deviations from normality (Shapiro-Wilk, 1965) and means of significant effects were compared using Student's t-LSD (Least Significant Differences) at the 95% confidence interval. All the above analyses were performed using SAS 9.2 Statistical Software, for continuous variables, means and standard errors were employed (SAS, 1999).

Chapter 4.

Results

4.1. **Introduction**

In this chapter, results are presented of the three objectives that included: evaluation of maturation rates and embryo development, evaluation of caspase-3 activity at different stages of embryos and detection of fragmented DNA at different stages of embryo life.

4.2. ***In vitro*** **oocytes maturation**

Different maturation temperatures (39, 41, 42 and 43 ˚C) were tested and the results are presented in table 4.1. Figure 4.1 and 4.2 shows oocytes matured at the four temperatures. Maturation of oocytes demonstrated no significant difference on the extrusion of polar body between 41 ˚C (60.0±1.2) and 39 ˚C (61.3±1.0). and no significance difference was observed on the non-polar body at both temperatures (table 4.1). Maturation of oocytes at incubation temperatures 42 ˚C and 43 ˚C showed significantly lower maturation rates and 43 ˚C thus was the lowest in polar extrusion and higher non-polar body was observed. The higher incubation temperatures (42˚C and 43˚) resulted in oocytes with disrupted cytoplasm and lower maturation rate.

Table 4.1 Maturation of cattle oocytes at four incubation temperatures :39, 41, 42 and 43˚C (MEAN ± SD)

Treatment	No. oocytes	Polar body (%)	Non-polar body (%)
41 ˚C	**201**	**60.0±1.2[a]**	**40.1±1.9 [b]**
42 ˚C	188	20.5±1.0[b]	60.3± 1.0[a]
43 ˚C	196	10.3±1.0 [b]	80.8 ±1.0 [a]
39 ˚C	**192**	**61.3±1.0[a]**	**39.1 ±1.5 [b]**

[a,b] value with different supercripts within columns differ significantly (P<0.05)

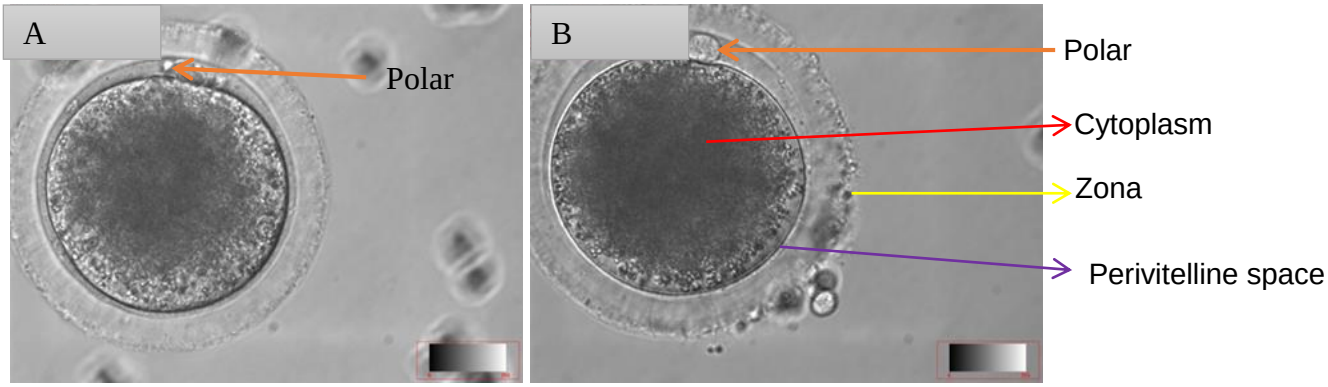


Figure 4. 1. (A) Matured oocytes from 41˚ C and (B) Matured oocytes from 39 ˚ C

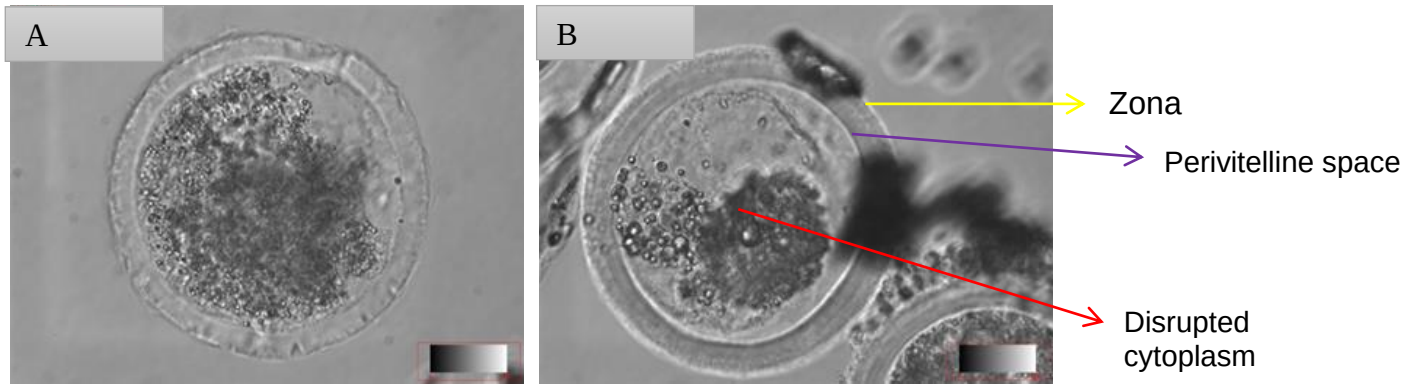


Figure 4. 2. Matured oocytes at (A) 42 ˚C and (B) 43 ˚C maturation temperature

4.3. ***In vitro*** **embryo production and nuclei cell analysis**.

Oocytes matured at 39 and 41 ˚C were selected for subsequent embryonic development as a result of higher extrusion of polar bodies compared to the other maturation temperatures (42 and 43 ˚C). There was no significant difference on the development rate of 2-4 cell and 8 cell embryos from both maturation temperature 39 and 41 ˚C (Table 4.2). Moreover, morula percentages {41 ˚C (19.0±14.16), 39˚ C (15.2 ± 4.8)} and blastocyst formation {39˚ C (11.4 ± 2.6) and 41 ˚C (11.2 ± 6.3)} also did not show any difference on both maturation temperatures. There was a higher significant ($P<0.05$) effect on the nuclei cell number with higher nuclei observed in blastocyst produced from oocytes matured at 39˚ C (133.2 ± 57.9) compared to 41˚ C (45.8 ± 11.2).

Table 4. 2 *In vitro* embryo development of matured oocytes at 39 ˚C and 41 ˚C incubation temperature (MEAN ± SD).

Treatment	No oocytes	2-4 cell (d2)	8 cell (d2)	Cleavage rate (%)	Morula (d5)	Blastocyst (d7)	Cell nuclei (n)
39 ˚C	398	65.0± 6.8[a]	39.2 ± 6.6[a]	72.0±22.70[a]	15.2 ± 4.8[a]	11.4 ± 2.6[a]	133.2± 57.9[a]
41 ˚C	395	62.4± 6.5[a]	35.0± 6.4[a]	60.2± 18.9[b]	19.0±14.16[a]	11.2 ±6.3[a]	45.8 ± 11.2[b]

[a,b] values with different superscripts within the rows differ significantly (P<0.05).d=number of days

Day 7 embryos from oocytes matured at two temperatures (39 and 41 ˚C) are illustrated in figure 4. 3

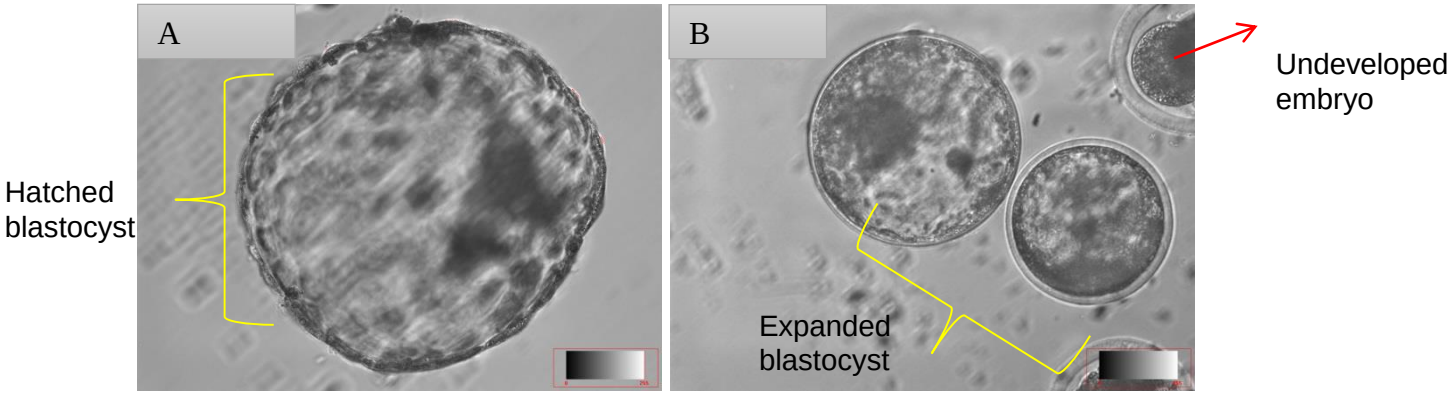


Figure 4. 3. (A) Day 7 Blastocyst from oocytes produced 39˚C and (B) Day 7 Blastocyst from 41˚C

Blastocyst stained with Hoechst 33323 from (39 and 41 ˚C) maturation temperatures are illustrated in figure 4.4.

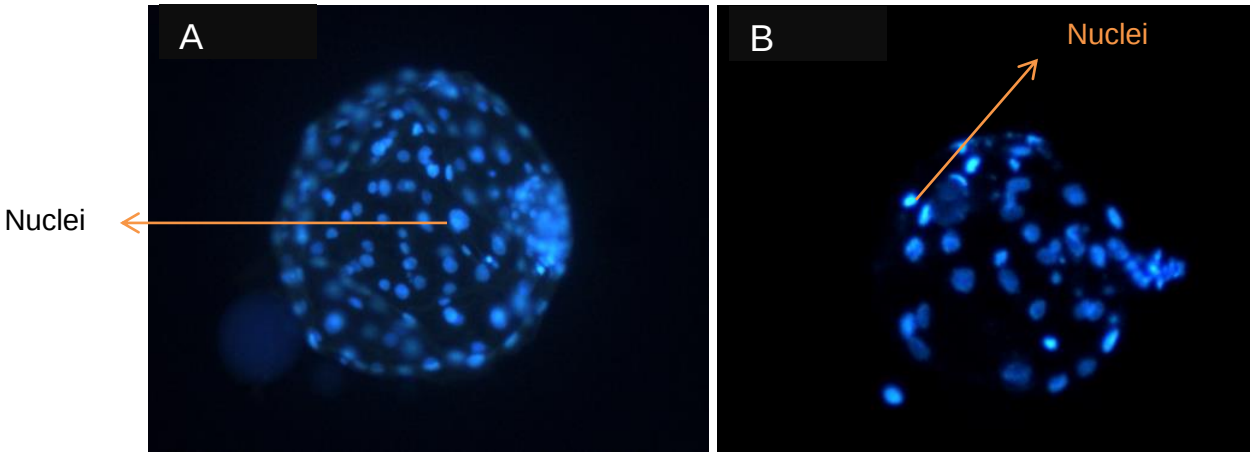


Figure 4. 4 (Hoechst 33323) Stained blastocyst from (A) 39 ˚ C and (B) 41˚ C

4.3. **Caspase- 3 activity of produced embryos from oocytes matured at two (39 and 41 ˚C).**

Caspase-3 activity results of 2-4 cell, ≤ 8 cell and blastocyst produced are indicated in table 4.3. Embryos at 2-4 cell showed no significant differences on caspase activity from oocytes matured at both 39 and 41 ˚C incubation temperatures. The optimal density values (OD) obtained were comparable on 2-4 cell embryos of oocytes incubated at 41 ˚C (0.016 ± 0.002) and 2-4 cell embryos from oocytes matured at 39 ˚C (0.014 ± 0.001). Moreover, the OD values for embryos ≤ 8 cell showed no significant difference on both 39 ˚C (0.022 ± 0.007) and 41 ˚C (0.032 ± 0.013). However, caspase-3 activity was found to be significantly higher on blastocyst from oocytes matured at 41 ˚C with the OD values (0.053 ± 0.005) compared to oocytes matured at 39 ˚C (0.037 ± 0.012).

Table 4. 3 Caspase activity at different cattle embryos stages produced in vitro embryo (MEAN ± SD)

Temperature	Control	2- 4 cell	≤ 8 cell	Blastocyst
39 ˚C	0.815± 0.049[a]	0.015 ± 0.001[b]	0.022 ± 0.007[a]	0.037 ±0.012[b]
41 ˚C		0.016± 0.002 [a]	0.032 ± 0.013 [a]	0.053 ± 0.005[a]

[a,b] values with different superscripts within the columns differ significantly (P<0.05)

4.4. **DNA fragmentation of produced embryos from oocytes matured at two (39 and 41 ˚C).**

Tunel assay results for DNA fragmentation on produced embryos are shown in table 4.4. There was evidence of DNA fragmentation observed in 2-4 cell embryos at 41 ˚C (6.5 ± 2.9) incubation temperature compared to 2-4 cell stage embryos at 39 ˚C (2.2 ± 1.2) temperature group. However, embryos ≥8 cell showed high significant difference (P< 0.05) at 41 ˚C (13.3 ± 3) compared to embryos at 39 ˚C (4.5 ± 1.9) and also on 2-4 cell (6.5 ± 2.9) embryos from the same temperature group (41 ˚C). The results showed no significant difference (P> 0.05) on the tunel positive between 2-4 cell (2.2±1.2) and ≥8 cell embryos (4.5 ±1.9) at the 39 ˚C incubation temperature. However, there was significantly low tunel negatives from ≥8 cell groups (7.7 ± 1.6) compared to 2-4 cell (18.3 ± 4.1) on embryos incubated at 41 ˚C. Moreover, the results demonstrated equal number of tunel negative (P >0.05) at 39 ˚C on 2-4 cell (18.2 ± 4.8), 8-16 cell (18.5 ± 2.7) and 2-4 cell embryos at 41 ˚C (18.3±4.1).

Table 4.4 DNA fragmentation using tunel assay in embryos matured at 39˚C and 41˚C (MEAN ± SD).

Temperature	Embryo group	Tunel positive (%)	Tunel negative (%)
39˚C	2-4 cell	2.2±1.2[c]	18.2± 4.8 [a]
	≥ 8 cell	4.5± 1.9 [bc]	18.5± 2.7 [a]
41˚C	2-4 cell	6.5± 2.9 [b]	18.3±4.1[a]
	≥8 cell	13.3± 3.1[a]	7.7± 1.6 [b]

[a,b,c] value with different supercripts within the rows differ significantly (P<0.05)

In figure 4.5 and 4.6 the stained 2-4 cell and ≥ 8 cell post DNA fragmentation at both temperatures are shown respectively.

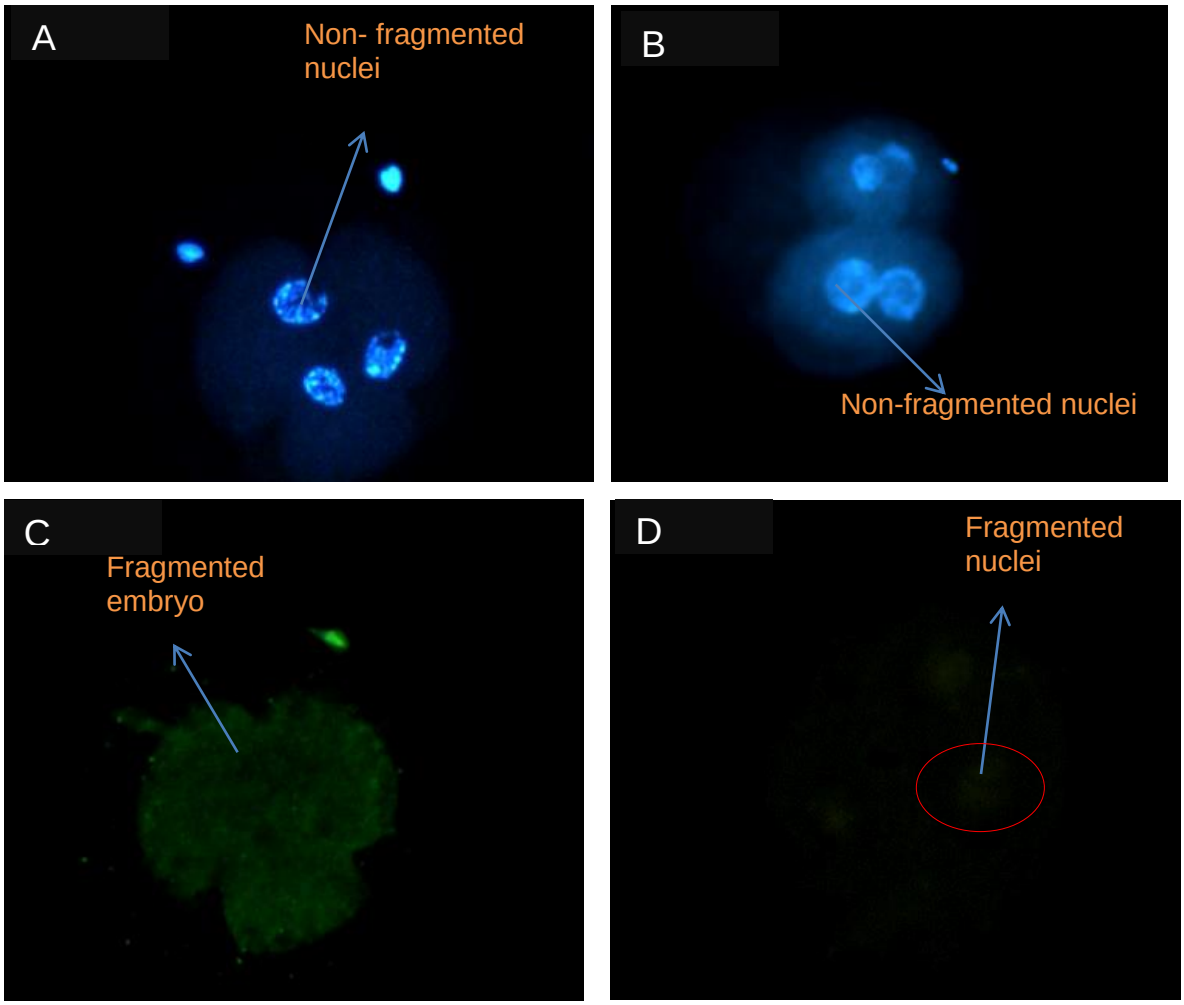


Figure 4. 5 (A) Non-fragmented 2-4 cell embryos from 41˚ C and (B) Non-fragmented 2-4 cell embryos from 39 ˚ C. (C) Fragmented embryos from 41 ˚ C and (D) Fragmented embryo from 39 ˚ C.

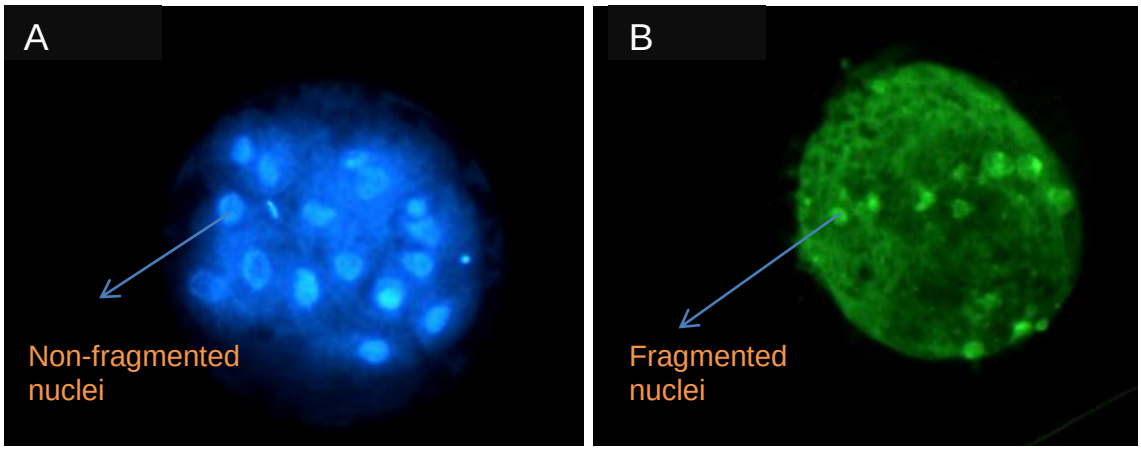


Figure 4. 6 (A) Non-fragmented ≥ 8 cell embryos from both 41 and 39 ˚ C (B) Fragmented ≥ 8cell embryos from both 41 ˚ C and 39 ˚ C

In table 4.5 the outcomes of post tunel index evaluated from blastocyst produced from 39 ˚C and 41 ˚C matured oocytes are shown. Oocytes incubated at 39 ˚C developed comparable number of blastocyst (12.0 ± 1.2) with oocytes matured at 41 ˚C (10.0 ± 1.4). However, the number of nuclei counted at both temperature groups resulted in significantly higher level of nuclei number (P<0.05) on the 39 ˚C (127.8 ± 53.4) incubation temperature compared to the 41 ˚C (46.2 ± 10.0). The 41˚C incubation temperature showed higher (P<0.05) percentage of fragmented DNA (21.8 ± 21.8) compared 39 ˚C (10.3 ± 4.8) tunel indexes (48.0 ± 8.2) than the 39 ˚C (9.7 ± 6.7) incubation temperature.

Table 4. 5 Tunel assay indexis on day 7 cattle blastocyst produced *in vitro* (MEAN ± SD).

Temperature	No blastocysts	Nuclei number	Tunel positive nuclei (%)	Tunel indexs (%)
39 ˚C	12.0 ± 1.2[a]	127.8 ± 53.4[b]	10.3 ± 4.8[b]	9.7 ± 6.7[b]
41 ˚C	10.0± 1.4[a]	46.2 ± 10.0[a]	21.8 ± 21.8[a]	48.0 ± 8.2[a]

[a,b] value with different supercripts within the columns differ significantly (P<0.05).

4.1.2 Figure 4.7 illustrate images of stained blastocyst for DNA fragmentation using tunel assay and Hoechst 33323.

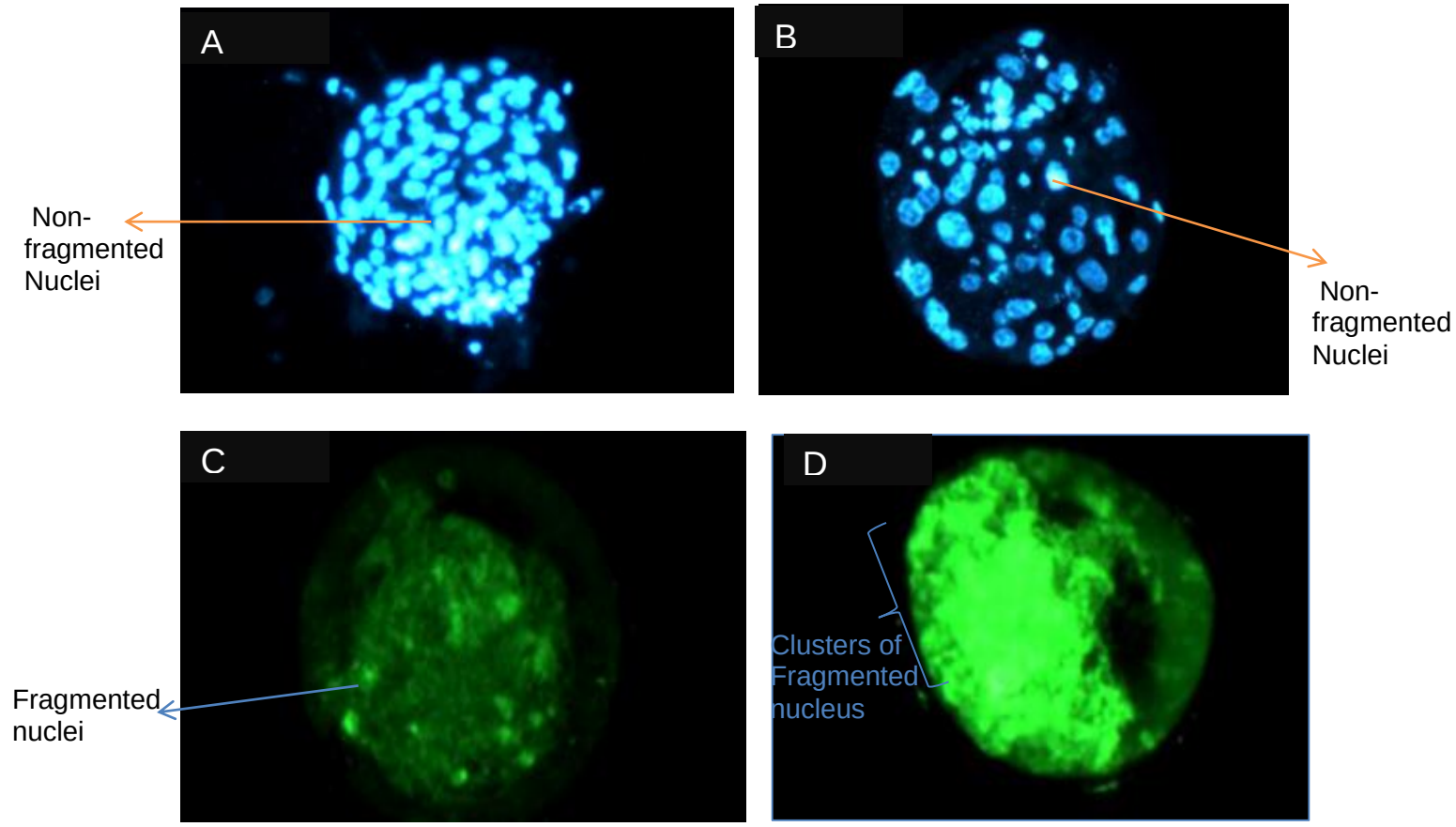


Figure 4. 7 (A & B) Non-Fragmented blastocysts from oocytes matured 39 ˚ C and 41 ˚ C, (C &D) Fragmented blastocysts from oocytes matured at both 39 ˚ C and 41 ˚ C.

Chapter 5.

Discussion.

5.1. Introduction

The present study of embryo production *in vitro* was designed to examine the effects of apoptosis on beef cattle embryos. Increased ambient temperatures and normal temperature on beef cattle oocytes during maturation was used to determine the effect of apoptosis on the normal *in vitro* development of embryos. The impact that the artificially environment of embryo production has on the produced embryos was of great importance in terms of cell apoptosis on produced embryos. The results obtained from this study were collectively gathered from oocyte maturation and embryo production, Caspase-3 activity and DNA fragmentation on *in vitro* produced embryos.

5.2. *In vitro* oocytes maturation

It has been documented that exposure of oocytes during maturation to high temperature at an earlier stage interferes with the process of oocyte maturation and may result in oocytes maturation failure. Roth & Hansen (2004) further highlighted, during maturation period, the temperature range of 40-41°C may induce apoptosis through DNA damage in oocytes before fertilization of the gametes. However, in the present study *in vitro* maturation resulted in comparable polar body extrusion between moderate heat-stressed (41 °C) oocytes with the non-heated (39 °C) oocytes. Moreover, literature state that, when cells are exposed to high temperature, toxic chemicals and other forms of stresses, the stress protein called heat shock proteins (HSPs) or are readily synthesized or induced (Kim *et al*., 2002). These proteins are essential for the maintenance of cellular homeostasis and protection against environmental insults. They assist in forcing other protein with incorrect folding to adopt proper conformation and folding, they further repair damaged occurred in cells due to various stressors (Rynkowska *et al.,* 2011). Therefore, this might have had an influence on the maturity survival of oocytes incubated at moderate high incubation temperature (41 °C) in this study.

The present study showed higher non maturation rates and disrupted cytoplasm at maturation temperatures of 42 and 43 °C. Conversely, Ju *et al*. (2005) and Roth & Hansen (2005), argue that the effect of heat stress or any other stress on oocytes damage depends on the severity of the stress and the oocyte thermotolerance. High temperature in different cell structures and organelles can results in damage that can be regain later through formation of newly transcript

in the cell or at some severe cases the damage can be irreversible. This confirms the irreversible damage observed in this study caused by heat shock (42 and 43 °C) in terms of dysfunctional cytoplasm compared to oocyte exposed to moderate heat shock (41 °C). In the study by Santos *et al*. (2010), it was reported that, heat stress decreases the ability of oocyte maturation in relation to the time of exposure to 41 °C temperature. The report further indicated that 70.7% of oocytes mature when incubated at 39 º C, while exposure of oocytes to thermal stress at 41 °C for 3, 6, 12, 18 and 24 hours, lead to 45.28%, 35.17%, 12.30%, 9.74% and 4.60% oocytes maturation respectively. Therefore, this shows that it is not only the severity of the stress that can contribute to cell damage but the time of exposure to stress might also have an influence as well.

Different species also respond differently on exposure to heat shock, according to Mortensen *et al.* (2010), equine oocytes are not sensitive to physiologically high temperatures (42 °C) for 2 or 4 h at the onset of IVM. However, in the present study maturation of cattle oocytes at 42 and 43 °C was affected and resulted in higher level of non-polar formation.

5.3. ***In vitro*** **embryo production**

The present study further investigated the survivability of embryos produced from oocytes matured at both (39 and 41 °C) maturation temperature groups. Similar to the maturation results, the study revealed comparable results on subsequent developmental stages of embryo life amongst the two maturation groups. According to Schrock *et al.* (2007), oocyte maturation at 41 °C does affect cleavage rate, subsequent embryo development after fertilization, nor the quality of developing blastocysts in the pre-implantation stage of the embryo life. These findings have been confirmed by Kim *et al*., (2002), Edwards *et al*., (2005), and Schrock *et al*., (2007), providing evidence that during the first part of meiotic maturation elevated temperatures has the ability to speed-up important development processes in the produced embryos. Furthermore, Silva *et al*. (2013), reported that the cell is able to activate cellular response in the presence of heat stress and therefore the response will minimize the damage effects course by the stressor hence, similar cleavage percentages were observed from oocytes exposed 41 °C and that of the 39°C oocytes in this study. The results provided by Roth & Hansen (2005), are also comparable with our results where oocytes incubated at 41 °C had similar morula percentages and blastocyst percentages with the non-heated oocytes. Roth & Hansen (2005), further highlighted that, incubation of oocytes at high temperature such as 40 °C during fertilization do not alter with cleavage of embryos and thus tends to increase the blastocysts rates compared with oocytes fertilized at 38 ˚C in cattle.

Oocytes and subsequent embryo development during heat stress is also considered to differ amounts breeds. The potential of oocytes development becomes detrimentally reduced in Bos Taurus when are collected and cultured *in vitro* during hot season *(*Friesian and Brown Swiss). The Bos indicus (Nguni and Brahman) cows (Silva *et al*., 2013) have an advantage and are not affected by as severely. This therefore explains the similarities on blastocyst rates on both 39 °C and 41°C in the present study since the ovaries were collected from Bos indicus cattle. Moreover, Bos indicus embryos shows more thermoregulatory ability during periods of heat stress than Bos taurus*.* In those studies, Brahman (Hernández-Ceren *et al.*, 2004) and Romosinuano (Hernández-Ceren *et al*., 2004) embryos were more resistant to heat stress 41 °C for 6 hours compared to Holstein (Paula-Lopes *et al*., 2003) and Angus embryos (Paula-Lopes *et al*., 2003). Similarly, Barros *et al*. (2006), Eberhardt *et al*. (2009) and Satrapa *et al*. (2011), reported that embryos of both pure and mixed Bos taurus breeds are susceptible to heat stress such as incubated at 41 ˚C for 12 hours than Bos indicus embryos. Moreover, there is evidence that Bos indicus and some Bos Taurus breeds (Senepol and Romosinuano) have a genetic disposition that allows their cells to have protective mechanism against from the effects of high temperatures (Satrapa *et al*., 2011).

5.4. **Nuclei cell analysis of produced blastocyst**

The results of embryo production on this study showed no significant differences on the blastocyst formation from both maturation temperature (39 and 41 °C). Thus, these findings further indicate that embryos from heat-stressed oocytes are capable of developing to more advanced stages in embryo production and may appear morphologically normal. Therefore, the day 7 blastocysts produced form this work was examined for nuclei cell number to morphologically examine the produced blastocyst. The results differed significantly ($P<0.05$) between nuclei cell count on 41 and 39 °C, where 41 °C had very low number of nuclei than the 39 °C group. The findings are similar to the work of Sakatani et *al*. (2004), where percentages of embryos developing to blastocyst stage after exposure to heat shock morphologically appeared normal, however with significantly low total nuclei cell number decreased by heat shock compared with the control. Ju *et al*. (2005), suggested that the reduction in the blastocyst total cell number may be due to a decrease in the trophectoderm. It is considered that heat shock at metaphase II of the oocyte life exerts a more deleterious effect on future trophectoderm cells (Ju *et al*., 2005). However, experiment work of Sakatani *et al*. (2015), revealed that embryos that reached the blastocyst stage which were exposed to heat stress at the early stages of the oocyte does not affect the total cell number of the trophectoderm cell and the inner cell mass ratio any differently compared to the none heat

shocked embryos during *in vitro* culture. Therefore, all these findings suggest that embryos that have passed through the effects of heat exposure might results in the reduction of cell nuclei number or restore a normal viability of nuclei cell number regardless of exposure to heat stress (Sakatani et *al*., 2004).

5.5. **Caspase -3 activity of produced embryos from oocytes matured at two (39 and 41 ˚C).**

It has been indicated that stresses due to high temperature on cattle embryos induces cell apoptosis (Rynkowska *et al.,* 2011; Boumela *et al.*, 2011) and that the *in vitro* culture environment itself causes cell apoptosis (Farin *et al*., 2010). Therefore, the *in vitro* produced embryos in this study were examined for the activity of caspase-3 because caspases are the main initiator of apoptosis through the release of the DNAse enzyme. The release of this enzyme breaks the DNA end and results in fragmentation of the DNA (Mehmet, 2000). In this study the optimal density values for caspase- 3 activity of the 2-4 cell embryos incubated at both temperatures differed slightly ($P<0.05$) with less amount of caspase activity. The results are similar to findings of Brad *et al*. (2007), where there was less caspase- 3 activity on 2-4 cell embryos from both 39˚ C and 41˚ C incubation temperatures. Results from both studies indicate that, the pro-apoptotic signals can be activated to initiate apoptosis at a later stage of embryo development after the embryonic genome activation. The caspase deoxyribonuclease and the resistance of mitochondrial depolarization at 2 cell results in inhibition of apoptosis or the presence of apoptosis at a lesser amount at 2 cell stage in cattle embryo (Hansen, 2015).

The balance between the pro- apoptotic and anti- apoptotic protein is important, however the combination of high amounts of ant-apoptotic protein with the low amounts of pro-apoptotic protein can results in mitochondrial depolarization (Hansen & Fear, 2011). Literature also show that, although the maturing oocyte can undergo apoptosis (Roth & Hansen 2005), apoptosis can be lost or at a minimum level at 2-cell stage during minor embryo genome activation and become visible between the 8 and 16-cell stages (Gjørret *et al*., 2007). Apoptosis response in cattle embryos in most cases occurs in abundance during the embryonic genome activation which is from 8-16 cell stage in cattle (Fear & Hansen, 2011; Silva *et al*., 2013). This might be that, DNA is highly methylated at the 2-cell stage and then becomes progressively more demethylated as development progresses until by the 8-16 cell stage when the embryo has low methylation and transcription activation (Dean *et al.,* 2001; Park *et al.,* 2006). Therefore, this could be reason for limited caspase activity- 3 observed from this study at 2-4 cell stage at both temperatures.

In the present work, embryos from ≥ 8 cell at both 39 and 41˚ C showed no difference in caspase -3 activity. These results are in contradiction with Jakob *et al*. (2007), where most of the caspase-3 activity staining was observed in the cytoplasm of single blastomeres in heat shocked embryos at 4 and 8 cell stages and in almost all heat shocked morulae and blastocyst (Jakob *et al*., 2007). The similarity of results observed in the ≥ 8 cell stage embryos on the current study might however be in line with the report gave by Silvia *et al*. (2009), stating that during preimplantation development, the cattle embryo may go through a period where it is resistant to pro- apoptotic signals. Brad *et al*. (2007), further reported that in cattle embryos mitochondrial membrane can became artificially depolarized by carbonyl cyanide 3-chlorophenylhydrazone (CCCP). In this case, caspase-9 and caspase-3 activation takes place but DNA fragmentation does not occur.

Silvia *et al*. (2009), further mention that, one possible explanation for DNA resistance to CAD may reside with the structure of DNA in the early preimplantation embryo. At the 2-cell stage, little transcription takes place and DNA is highly methylated. DNA demethylation occurs over the next several cleavage divisions. Thus, the stage of development at which susceptibility to apoptosis is acquired (8–16-cell stage) is also a time when DNA methylation is reduced and transcription is activated (Silvia *et al*., 2009). Given that blastocyst produced from oocytes matured at 41˚C in this study showed higher caspase-3 activity at 39˚ C was in contrast with the findings from Roth & Hansen, (2004). They reported that the proportion of blastocyst from both 39 and 41 ˚C showed no significant difference on the caspase-3 activity where the least squares means for caspase activity were 21. 6 ± 1.6 vs. 19. 6 ± 1.6 respectively. However, the higher presence of caspase-3 activity on blastocyst in this study agrees with the report gave by Bryla *et al*. (2011), who mentioned that, as culture time increases more percentage of expanded blastocysts reveals more DNA fragmentation nuclei and activity of caspase-3 on embryos. Bryla *et al*. (2011), further mentioned that the longer period of *in vitro* culture increases the more percentage of embryos show DNA fragmentation and caspase- 3 activities.

5.6. **DNA fragmentation of produced embryos from oocytes matured at two (39 and 41 ˚C).**

Due to the importance of DNA fragmentation by caspase-activated DNase (CAD) through activation of caspase activity, it was important to evaluate morphological changes on preimplantation embryo development in the present study.

In this study, embryos at 2-4 cell stage of development showed significantly fewer positive apoptotic cell nuclei's at both temperatures 39 and 41˚ C compared to embryos at greater ≥ 8 cell stage. Thus, the finding consequently suggests that the activity release of DNase from activity of caspase in 2-4 cell embryos is at minimum, hence fewer DNA fragmentation in 2-4 cell stage. The apoptotic machinery is present in 2-cell embryo but the mitochondria are resistant to apoptotic signals following apoptotic stimulus. This however, suggests that there is a developmental regulation of apoptosis at the level of the mitochondria (Fear *et al*., 2011). Moreover, cattle embryo remains refractory to apoptotic stimuli until the 8- to 16-cell developmental stage, hence in the present study more tunel positive nuclei's were observed from embryo ≥ 8 cell than 2-4 cell embryos. Brad *et al*. (2007), repeated that both treated and non-treated embryos at the 2-4 cell stage of development represent less amount of TUNEL positive nuclei regardless of the induction of caspase initiation factors such as the procaspase-9 and procaspase-3 and therefore the DNA will also not be fragmented.

Apoptotic cell nuclei may also not become affected by stimulus such as heat shock in 2-4 cell stages of embryos. According to the findings of Sakatani *et al*. (2013)**,** heat shock exposure did not affect the percentage of inseminated oocytes that cleaved or that reached the 2- to 4-cell stage on day 2 after insemination**.** Elevated temperature does not affect the 2 cell stage or the 4 cell stage nuclei to become TUNEL positive, however, the higher incubation temperature at this stage will affect a fraction of the blastomere to became TUNEL positive at a later stage of the embryo development, mainly the 8-16 cell stage (Brad *et al*., 2007). Hence more blastocysts were fragmented at 41 than 39˚ C in the present study.

In conclusion, these results suggest that it is important to perform morphological quality control of pre-implantation embryos, as well as sign of apoptosis. Quality control of *in vitro* embryo production may results in improvement of pregnancy rate of these embryos and lead to more effective implantation and application of IVP system.

CHAPTER 6

Conclusion

Embryo quality assessment is extremely important in case of embryo transfer but it also serves as a tool to evaluate the efficiency of *in vitro* embryo production systems. The presence of apoptotic cells has been identified as a negative embryo quality parameter, therefore evaluation of apoptosis will assist in selecting potential embryos for embryo transfer and successful implantation rate in South Africa.

Maturation results showed similar extrusion of polar bodies between 39 and 41 ˚C, with poor results at 42 and 43 ˚C showing low number of polar body present. The similarity of developmental rates of embryos in all stages post maturation suggested that both maturation temperatures (39 and 41 ˚C) may result in embryo development, however the blastocyst quality of oocytes matured at 41 ˚C was compromised, resulting in very low nuclei cell number compared to the embryos from 39 ˚C maturation temperature.

Apoptotic evaluation of the produced embryos from both temperature groups (39 and 41 ˚C) showed comparable optimal density values of caspase-3 activity on 2-4 cell and ≥ 8 cell embryos from both maturation groups. However, blastocyst produced from 41 ˚C maturation group showed higher optimal density values than those produced from oocytes matured at 39 ˚C. furthermore, DNA fragmentation results revealed a higher presence of fragmented DNA in embryos from oocytes matured on 41 ˚C compared to 39 ˚C maturation temperature in all the developmental stages. The Tunel indexes also showed higher positive percentage in blastocysts from the 41 ˚C maturation group compared to the 39 ˚C group. The project showed presence of apoptosis in both temperature groups on *in vitro* embryo production, however at small amounts in the normal *in vitro* temperature (39 ˚C), therefore, for healthy production of cattle embryos the *in vitro* embryo production system in South African laboratories requires supplementation of caspase inhibitors on *in vitro* production mediums to block the caspase reaction and improve implantation rates post transfer.

It has generally been accepted that culture conditions can have a major influence on embryo quality and it has also been shown that adequate changes of the culture environment can prevent or minimize the occurrence of apoptosis during embryo culture *in vitro*. Therefore, it is recommended that future studies commence on evaluation of pro and anti-apoptotic genes where pro-apoptotic genes promotes apoptosis post stimulus (Bax,Bcl-XS, Bak, Bad, Bik and

Bid) while anti-apoptotic genes (Bcl-2, Bcl-XL, Bcl-W, Mcl-1, A1) aids to prevent cytochrome *C* release which cleaves with the caspases to initiate apoptosis. Evaluation of these genes will aid in monitoring the activity of the caspases in the mitochondrion. The ant- apoptotic gene will assist the mitochondrion to block the release of caspases for the initiation of apoptosis. This will then generate molecular information on the produced embryo. Therefore, both the knowledge of morphological and genetic information of the embryo will then result in healthier embryos produced *in vitro*.

The results from this study provide baseline information of factors such as temperature, Caspase activity and DNA fragmentation in cell apoptosis on *in vitro* produced beef cattle embryos in South Africa. Further study can now follow where genetic mechanism can be investigated associated with cell apoptosis in these embryos.

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