27 ABSTRACT

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29 Telomerase, a ribonucleoprotein, has been described as an essential component of highly
30 proliferative cells as it stabilizes telomeres in order to avoid cellular senescence. In most
31 mammalian species, telomerase activity is present in germ cells but not in somatic cells.
32 The objective of this study was to examine the telomerase activity in swamp buffalo
33 immature and mature oocytes, and preimplantation stage embryos derived from in vitro
34 fertilization (IVF), somatic cell nuclear transfer (NT) and parthenogenetic activation (PA).
35 Immature and mature oocytes, and embryos at 2-4-cell, 8-16-cell, morula and blastocyst
36 were collected and the telomerase activity was assayed by using a Telomerase PCR
37 ELISA kit. Telomerase activity was detected in all developmental stages evaluated from
38 immature oocytes to blastocyst stage embryos. Telomerase activity was detected in higher
39 amounts in immature compared with mature oocytes ($P < 0.05$). Embryos derived from
40 NT showed a profile of telomerase activity similar to that of IVF. In IVF and NT
41 embryos, telomerase activity was low in the 2-4-cell and 8-16-cell stages, the activity was
42 significantly increased ($P < 0.05$) at the morula stage, reaching highest level at the
43 blastocyst stage. In PA embryos, low level of telomerase activity was detected from the
44 2-4-cell to the morula stage, and the highest level of telomerase activity were found at the
45 blastocyst stage. Telomerase activity in NT blastocysts is higher than that derived from
46 IVF and the activity is highest in PA blastocysts. These results suggest that the successful
47 reprogramming of telomerase activity in buffalo NT embryos follows a similar pattern to
48 that in embryos derived from IVF and PA.

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50 Key Words : telomerase activity, buffalo, nuclear transfer, in vitro fertilization,
51 parthenogenetic activation

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INTRODUCTION

Telomeres, the specialized DNA sequences located at the ends of eukaryotic chromosomes, consist of evolutionarily conserved repetitive noncoding DNA sequence (TTAGGG) that functions as stabilizing elements for the chromosomes during cell divisions (Preston, 1997). Telomeres have been observed to associate with nuclear envelope and may be necessary for initiation of homologue alignment during meiosis (Cooper et al., 1998) and chromosome segregation during mitosis (Yu et al., 1990). Telomeric DNA is not completely synthesized by conventional DNA polymerase during cell replication and also loses 50-200 base pairs with each cell division (Olovnikov, 1973; Greider and Blackburn, 1985). Telomere shortening correlates with the numbers of cell division and when the telomere length reaches a critical limit, it signals the cells to undergo replicative senescence, in which state the cells develop a characteristic morphology of large and flat and stop dividing (Faragher and Kipling, 1998).

Telomerase is a ribonucleoprotein that compensates for the loss of telomeric DNA by adding repeated sequences to the chromosomal ends using its intrinsic RNA component as a template for DNA synthesis (Dahse et al., 1997). The telomere hypothesis suggests that telomerase activity is high in embryonic cells and that it decreases in somatic tissues during development and differentiation. Telomerase activity has been detected in germline cells (Wright et al., 1996; Eisenhauer et al., 1997), embryonic cells (Eisenhauer et al., 1997; Betts and King, 1999; Xu and Yang, 2000; Wright et al., 2001), embryonic stem cell lines (Thomson et al., 1998), cancer and immortalized cell lines (Kim et al., 1994; Counter et al., 1994). High levels of telomerase activity were detected in rat oocytes from antral and preovulated follicles, but activity was significantly lowered in ovulated oocytes (Eisenhauer et al., 1997). Reports in cow (Bette and King,1999) and human (Wrigth et al.,

2001) demonstrated that telomerase activity could be detected in all developmental stages starting from immature oocyte through blastocyst stage. Telomerase activity decreases gradually during early cleavage stage but increases at the morula and blastocyst stages. Recently, telomerase activity was also detected in bovine embryos derived from NT and PA (Xu and Yang, 2001). They found that telomerase activity in cloned embryos follows a similar pattern to that in the PA and IVF embryos, suggesting the successful reprogramming of telomerase after NT. Most of the previous studies used telomeric repeat amplification protocol (TRAP) method, in which the telomerase reaction product is amplified by polymerase chain reaction (PCR) to assay telomerase activity as described by Kim et al. (1994). However, the TRAP assay provides full sensitivity only when used with a radioactive label, and it requires visualization of results by autoradiography after gel electrophoresis, which is both hazardous and time consuming. Obyashiki et al., (1996) developed a nonradioactive procedure using fluorescence-labeled primers and automatic DNA sequencing to detect and quantitate telomerase activity. Furthermore, the telomerase activity detection in RKO colorectal tumor cells by standard radioactive TRAP, as well as by the PCR ELISA (a nonradioactive ELISA-based assay) showed that the results obtained with both procedures correlate well (Smilenov et al., 1997). The Telomerase PCR ELISA provides a way to perform a highly sensitive photometric enzyme immunoassay for the detection of telomerase activity, using nonradioactive ELISA technique.

Our previous reports demonstrated that buffalo fetal fibroblast could be reprogrammed after transfer into enucleated buffalo or bovine oocytes resulting in the production of cloned buffalo blastocysts (Kitiyanant et al., 2001; Saikhun et al. 2002). However, there has been no study on telomerase reprogramming of buffalo oocytes and embryos during early development. Moreover, investigating the dynamics of telomerase activity in buffalo

embryos produced by IVF or PA could serve as good developmental controls for NT studies. The objective of the present study was to investigate the dynamics of telomerase in swamp buffalo oocytes and preimplantation stage embryos derived from NT, IVF and PA by using the Telomerase PCR ELISA technique. Furthermore, the patterns of telomerase activity in embryos derived from those three systems were compared.

MATERIALS AND METHODS

Chemicals

All chemicals in this study were purchased from Sigma Chemical Company (Sigma, St. Louis, MO) unless stated otherwise.

Oocyte Collection and In Vitro Maturation

Buffalo oocytes were collected and matured in vitro by the method previously described (Pavasuthipaisit et al., 1992). Cumulus oocyte complexes (COCs) from abattoir ovaries were collected by aspirating the antral follicles (2 to 6 mm) using a 18-guage needle containing TALP-HEPES. After being washed for three times, COCs were morphologically assessed under a stereomicroscope (x 200) and only oocytes with compact and homogeneous cytoplasm were selected for in vitro maturation (IVM). All selected COCs were cultured in 50 µl drops of maturation medium (TCM 199) (Gibco, Grand Island, NY) supplemented with 10 % fetal calf serum (FCS) (Hyclone Laboratories, Inc, Logan, UT), 0.2 mM pyruvate and 5 µg/ml FSH in a humidified atmosphere of 5 % CO_2 at 39 °C. After culturing for 22 hr, mature oocytes were subjected to IVF, NT or PA. Immature (germinal vesicle) and mature (metaphase II, MII) oocytes were collected and stored at - 80 °C until telomerase activity was assayed.

In Vitro Fertilization of Buffalo Oocytes

In vitro production of buffalo embryos was based on the procedures reported in our previous study (Pavasuthipaisit et al., 1992) with minor modifications. Frozen ejaculated semen were thawed at 37°C and sperm was prepared by using swim up technique. Sperm

samples (0.25 ml) were layered under 1 ml of TALP- glucose free medium in centrifuge tubes. After 1 hr of incubation under a humidified atmosphere of 5 % CO_2 at 39 °C, the top portion (0.85 ml) of the medium was removed from the tube. Sperm suspension was centrifuged at 500g for 20 min, and the resulting sperm pellet was resuspended to a final concentration of 50 x 10^6 cells/ml in TALP for in vitro fertilization. A 10 µl aliquot (5 x 10^5 cells/drop) of sperm was placed in a culture dish with 50 µl of glucose free-TALP containing 10 µg/ml heparin to facilitate capacitation. Spermatozoa were incubated for 2 hr under a humidified atmosphere of 5 % CO_2 at 39 °C. Ten in vitro matured COCs were added to each fertilization drop containing capacitated sperm. After 18 to 20 hr of insemination, presumptive zygotes were removed from fertilization drops and transferred to in vitro culture.

Somatic Cell Nuclear Transfer

Cloned buffalo embryos derived from somatic cell NT were obtained as described previously (Kitiyanant et al., 2001). Briefly, after being cultured in maturation medium for 22 hr, buffalo oocytes were denuded by pipetting in TALP-HEPES containing 0.2 % hyaluronidase. Oocytes with an extruded first polar body (MII) were selected for enucleation and used as recipient cytoplasm in NT procedure. The MII oocytes were placed in TALP-HEPES supplemented with 10 % FCS, and 7.5 µg/ml cytochalacin B and were enucleated by aspirating the first polar body and the MII plate with small volume of surrounding cytoplasm. Successful enucleation was confirmed by Hoechst 33342 staining and observed under ultraviolet light. Buffalo fetal fibroblasts were obtained from a 40-day old fetus, and cultured in DMEM supplemented with 10 % FCS and used for NT between passages 2 and 6 of the culture. They were synchronized to enter into presumptive G0 by culturing in DMEM supplemented with 0.5 % FCS for 3 to 5 days prior to the NT

procedure. Immediately prior to NT, single cell suspension was prepared from donor cells by trypsinization using 0.25 % trypsin-EDTA. Individual donor cells were transferred into perivitelline space of the enucleated oocytes. Nuclear transfer units were washed and incubated in a fusion medium (0.3 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4.7H_2O$, 0.5 mM HEPES and 0.05% fatty acid-free (FAF) BSA) for 1 min. Fusion was performed by placing NT units in a fusion chamber between two platinum electrodes 0.2 mm apart and overlaid with fusion medium. Cell fusion was induced by two DC electrical pulses of 2.1 kV/cm for 20 μsec (1 sec interval). After being co-cultured with buffalo rat liver (BRL) cells in TCM 199 supplemented with 10 % FCS for 2 hr, the fusion rate was determined. Fused NT embryos were activated by exposure to 5 μM calcium ionophore A23187 for 5 min followed by incubation with 2 mM 6-dimethylaminopurine (6-DMAP) in culture medium for 4 hr under a humidified atmosphere of 5 % CO_2 at 39 °C and subjected to in vitro culture.

Parthenogenetic Activation of Buffalo Oocytes

The PA procedure was performed as described previously (Liu et al., 1998). Briefly, after being cultured in maturation medium for 22 hr, buffalo oocytes were denuded by pipetting in TALP-HEPES containing 0.2 % hyaluronidase. The oocytes with an extruded first polar body (MII) were parthenogenetically activated by exposure to 5 μM calcium ionophore A23187 for 5 min followed by incubation with 2 mM 6-DMAP in culture medium for 4 hr under a humidified atmosphere of 5 % CO_2 at 39 °C subjected to in vitro culture.

209 Embryo Culture and Collection

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211 The IVF, NT and PA embryos were cultured under the same systems. They were co-
212 cultured with BRL cells in 50 µl of TCM 199 supplemented with 10 % FCS under a
213 humidified atmosphere of 5 % CO_2 at 39 °C. After culturing for 2 days, the embryos were
214 assessed for the first cleavage completion and transferred to 50 µl drops of TCM 199
215 containing buffalo oviductal epithelial cells. Cleaved embryos were cultured under the
216 same system as with BRL cells for 7 days. Embryonic development to the 2-4-cell, 8-16-
217 cell, morula and blastocyst stages were recorded on Days 2, 3, 5 and 7 of in vitro culture,
218 respectively. At each stages, embryos were collected, washed twice in PBS and then
219 frozen at -80 °C until the telomerase activity was assayed.

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221 Telomerase Activity Assay

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223 The Telomerase PCR ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany)
224 was used to measure the telomerase activity of buffalo oocyte and embryo. The original
225 TRAP method described by Kim et al. (1994) was modified.

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227 Preparation of cell extracts

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229 Cell extracts from oocytes or embryos were prepared as described by the manufacture
230 using lysis reagent provided in the kit. Briefly, pools of 5 frozen (-80 °C) immature and
231 mature oocytes or embryos at each stages were lysed in pre-cooled 200 µl of lysis reagent
232 for 60 min and cell extracts were centrifuged at 16000 g for 2 min at 4 °C. The 175 µl of
233 supernatant was removed and the cell extracts were measured for the protein

234 concentration using spectrophotometer and immediately used for the TRAP reaction or
235 shock freeze aliquots in liquid nitrogen and stored at -80° C until analysis.
236
237 Telomeric Repeat Amplification Protocol (TRAP)
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239 Telomerase essentially requires integrity of its internal RNA component of the
240 telomerase-specific primer. Therefore, preincubation of the cell extracts with RNase
241 (DNase-free) will fully destroy telomerase activity contained in the extracts and offers as a
242 negative control. Positive control was supplied with the kit. Positive control cell extract
243 was prepared from immortalized telomerase - expressing human kidney (293-cell). One
244 microliter of low (0.001 amol/μl) or high (0.1 amol/μl) positive control DNA template
245 (TS8) was mixed with 1 μl of lysis reagent and nuclease-free water was added to a total
246 volume of 50 μl. For each sample to be tested and the control template, 25 μl of reaction
247 mixture (biotinylated telomerase substrate P1-TS, optimized anchor-primer P2,
248 nucleotides, and Taq DNA polymerase) and 5 μl of the internal standard (IS) were
249 transferred into a PCR reaction tube. One microgram total protein of cell extracts was
250 added into PCR tubes. The reaction tubes were incubated at 25 °C for 30 min, then
251 telomerase was inactivated by heating at 94 °C for 5 min. Samples were then subjected to
252 30 PCR cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 90 sec.
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254 Hybridization and ELISA
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256 An aliquot of the PCR product was denatured in 20 μl of denaturation reagent containing
257 less than 0.3% sodium hydroxide and incubated at 15-25° C for 10 min The mixture was
258 transferred into a streptavidin-coated 96-well plate and hybridized with a digoxigenin
259 (DIG) labeled, telomeric repeat specific detection probe After shaking at 37° for 2 hr, the

hybridization solution was removed and washed each well 3 times with 250 µl of washing buffer for a minimum of 30 sec each and then removed washing buffer carefully. One hundred microliters of anti-DIG conjugated horseradish peroxidase (HRP) was added per well, covered with a cover-foil and incubated at room temperature for 30 min while the plate was shaking at 300 rpm. The solution was removed and rinsed 5 times with 250 µl washing buffer into each well for a minimum of 30 sec each and then carefully removed the washing buffer. The amount bound to DIG was determined by the addition of 100 µl per well of TMB (3,3',5,5'-tetramethyl benzimide) substrate solution prewarmed to room temperature, covered the wells with foil and incubated for color development at room temperature for 10-20 min while shaking at 300 rpm. Without removing the reacted substrate, 100 µl of stop reagent containing less than 5% sulfuric acid was added into each well to stop color development. The absorbance of the samples was measured at 450 nm using an ELISA microtiter plate reader within 30 min after addition of the stop reagent. The experiments were replicated five times. For each replication, each sample was assayed at least twice and an average value was taken. The level of telomerase activity in each sample was determined by comparing the signal from the sample to the signal obtained using a known amount of a control template. Relative telomerase activity (RTA) of each sample was calculated using the following formula :

$$RTA = \frac{(A_S - A_{S0} / A_{S,IS})}{(A_{TS8} - A_{TS8,0}) / A_{TS8,IS}}$$

A_S : absorbance of sample

A_{S0} : absorbance of sample of RNase treated sample

$A_{S,IS}$: absorbance of internal standard (IS) of the sample

A_{TS8} : absorbance of control template (TS8)

$A_{TS8,0}$: absorbance of lysis buffer

$A_{TS8,IS}$: absorbance of internal standard (IS) of the control template (TS8)

286 Statistical Analyses

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288 Data of the telomerase assay experiment was collected from five replicated on different
289 pooled-oocyte or -embryo samples (n = 25). Analysis of variance (one-way ANOVA) was
290 used to determine any significant difference between RTA of oocytes and embryos at each
291 developmental stages. The relationship of relative telomerase activity and dilution of
292 positive cell extracts was subjected to regression analysis. Difference between treatments
293 were considered statisstically significant at $P < 0.05$.

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RESULTS

Telomerase Assay Verification

To verify that the Telomerase PCR ELISA assay kit is suitable to detect telomerase activity, we first determined the telomerase activity in cell lines known to express telomerase activity and those known to lack the activity. As expected, telomerase activity was found in high levels in the 293-cell, KB-cell (human carcinoma cell line) and buffalo ES-like cell (established in our laboratory) but having low level in buffalo fetal fibroblast and RNase treated 293-cell (Fig. 1). A subsequent experiment was examined a dose-response relationship between telomerase activity and amount of 293-cell extract. Figure 2 clearly shows a dose response resulting from progressive dilutions. A linear relationship was found between the telomerase activity and dilution ($r = 0.93$). These results indicated that the Telomerase PCR ELISA assay kit is a suitable method for quantification of telomerase activity

Telomerase Activity in Buffalo Embryos

Samples were taken to determine telomerase activity in all developmental stages from immature oocytes through blastocyst stage embryos. For each replication, pooled-oocyte or -embryo sample ($n = 5$) was assayed at least twice and average value was taken. Telomerase activity was presented in relatively high amounts in immature and decreased significantly ($P < 0.05$) in mature oocytes (Fig. 3). Telomerase activities in IVF-derived embryos at different stages are shown in Figure 4. Low levels of telomerase activity were detected in the 2-4-cell stage and the 8-16-cell stage embryos. The activity was significantly increased ($P < 0.05$) at the morula stage and was highest at the blastocyst

338 stage. A similar pattern of telomerase activity with IVF embryos was also detected in the
339 NT system (Fig. 4), telomerase activity having low level from the 2-4-cell to 8-16-cell
340 stages and increasing significantly (P · 0.05) in the morula stage and reaching the highest
341 level in the blastocyst stage (P · 0.05). In the PA system (Fig. 5), telomerase activity was
342 low and not significantly different from the 2-4-cell to the morula stages. A significantly
343 increased (P · 0.05) activity was detected at the blastocyst stage.
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345 A direct comparison of telomerase activity among the various stages, from the 2-4-cell to
346 the blastocyst stages of buffalo embryos produced by IVF, NT and PA systems, is shown
347 in Figure 6. There were no differences in telomerase activity of embryos at the 2-4-cell
348 and the 8-16-cell stages among three methods used for embryo production. Activity in the
349 morula stage of embryos produced by IVF and NT systems was significantly higher than
350 that produced by PA system. Highest telomerase activity was found in the blastocysts
351 from all three systems but telomerase activity in blastocysts from the PA and NT systems
352 appeared to be higher (P 0.05) than those the IVF
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DISCUSSION

The present study demonstrated that telomerase activity could be detected in buffalo oocytes through all developmental stages of IVF, NT or PA embryos. Our results confirmed the previous findings in rat oocytes and embryos, in which telomerase activity decreased from high levels in oocytes from early antral and preovulated follicles to lower level in the ovulated oocytes (Eisenhauer et al., 1997). A high level of telomerase activity in the immature oocytes was also detected in the cow (Betts and King, 1999) and recently in human (Wright et al., 2001). This high level of telomerase activity detected in immature oocyte and decreased in mature oocyte suggests a possible role of telomerase during meiosis of female germ cells in buffalo. It has been reported that during germinal vesicle breakdown process, the rates of protein and RNA synthesis declined dramatically (Wassarman and Albertini, 1994). Moreover, as much as one half of the polyadenylated RNA accumulated during oocyte growth was either degraded or deanylated during meiosis (Wassarman and Albertini, 1994). These biochemical changes may be responsible for the decrease of telomerase activity in oocyte maturation process.

In IVF system, the telomerase activities decreased gradually from immature oocytes to the 8-16-cell stage embryos. Enzyme activity increased again at the morula stage and reached its highest level at the blastocyst stage. Our findings are in agreement with the previous studies in bovine (Betts and King, 1999; Xu and Yang, 2000) and human embryos (Wright et al., 2001). Betts and King (1999) reported that relative telomerase activity decreased during bovine oocyte maturation and subsequent development to the 6-8-cell stage but significantly increased at the morula and blastocyt stages. On the other hand, Xu and Yang (2000) demonstrated that telomerase activity was increased after fertilization and then decreased gradually until the embryos reached the 8-cell stage. Telomerase

activity increased again after 8-cell stage and reached its highest levels in the blastocyt stage. Those authors suggested that a dramatic changes of telomerase activity in bovine embryos coincides with the maternal-zygotic transition during early embryonic development. In many species, the timing of the in vitro developmental block reportedly coincides with the stage when the regulation of embryo development switches from control by maternal proteins to the control by embryonic gene products (Telford et al., 1990). Our previous findings demonstrated that in vitro developmental block occurred in 8-16-cell stage during in vitro culture of buffalo IVF system (Pavasuthipaisit et al., 1992). The maternal proteins including telomerase gradually decrease during early cleavage until approximately the 8-16-cell stage. Once embryonic gene transcription starts, new telomerase proteins are synthesized and this will account for the increase of telomerase activity after the 8-16-cell stage as demonstrated in our study and others.

Our results demonstrated that telomerase activity in NT embryos was up-regulated as early as the morula stage and reached highest levels at the blastocyst stage which was similar to the IVF system. These findings suggested a successful reprogramming of telomerase in buffalo NT embryos reconstructed with fetal fibroblast which displayed low level of telomerase activity. High levels of telomerase activity were detected in reconstructed day 7 embryos tested by TRAP assay, whereas the bovine fibroblasts used as donor cells in the NT experiments were negative for telomerase activity (Lanza et al., 2000). Our results are consistent with recently report by Xu and Yang (2001) who demonstrated that up-regulation of telomerase activity in bovine NT embryos began as early as the morula stage. Another study by Betts et al. (2001) found that reprogramming of telomerase activity in bovine NT embryos was observed at the blastocyst stage which was delayed when compared with fertilization-derived embryos, in which high telomerase activity was observed after activation of the embryonic genome at the 8-16-cell stage

In PA embryos, low level of telomerase activity was detected from the 2-4-cell to the morula stage but the activity was significantly increased at the blastocyst stage. A direct comparison of telomerase activity in buffalo embryos produced by different procedures indicated that telomerase activity detected in the PA blastocysts was higher than that derived from IVF and NT. The reasons for higher telomerase activity in PA blastocysts than in IVF and NT blastocysts are unclear. Higher levels of telomerase in PA blastocysts when compared to those produced by IVF and NT was also reported in bovine (Xu and Yang, 2001). They suggested that the lower level of telomerase activity in NT blastocysts might come from a loss of cytoplasm in the enucleation steps or possibly from the variance between embryos, whereas the lower telomerase activity in IVF blastocysts might come from the nonmaternal genes that could have been actually suppressed the telomerase gene expression and, hence, reduced the telomerase activity. Chian and Sirard (1996) found that bovine oocyte activation by sperm and PA induced different cytoplasmic responses for protein synthesis.

Additionally, we also found that telomerase activity detected in NT blastocysts was higher than that derived from IVF blastocysts which differed from the previous report by Xu and Yang (2001) who demonstrated that telomerase activity in bovine NT blastocysts was lower than IVF. This discrepancy may come from species difference or from different NT techniques employed or from donor cell types. The reason for higher telomerase activity in NT blastocysts than that in IVF blastocysts is unknown. The lower telomerase activity in IVF blastocysts may be due to the suppression of telomerase gene by sperm factors as suggested by Xu and Yang (2001). At the same time, the telomerase gene from transferred somatic nuclei may be stimulated by factors in ooplasm, hence, higher the telomerase activity in NT system.

When the data on a per cell basis of embryos were analyzed, we found telomerase activity declined gradually from the immature oocyte through the blastocyst stage in IVF, NT and PA embryos. The similar declined of telemerase activity on a per cell basis have been reported in bovine (Xu and Yang, 2000) and human (Wright et al., 2001) IVF embryos. The reasons of these patterns is unkhown. Furhter studies are needed to examine the underlying mechanisms.

In summary, telomerase activity has a trend involving an increase from the mature oocyte to morula stage in both NT and IVF systems, whereas in PA system similar levels of the activies are present until the morula stage but all three types of embryos have the highest levels of telomerase activity at the blastocyst stage. These results demonstrated that telomerase plays an important role in oocyte maturation and embryonic development and there is successful reprogramming of telomerase activity in embryos produced by NT. Further investigations are required to examine the relationship between telomerase activity in preimplantation stage embryos and the survival of embryos after transfer into surrogate mother.

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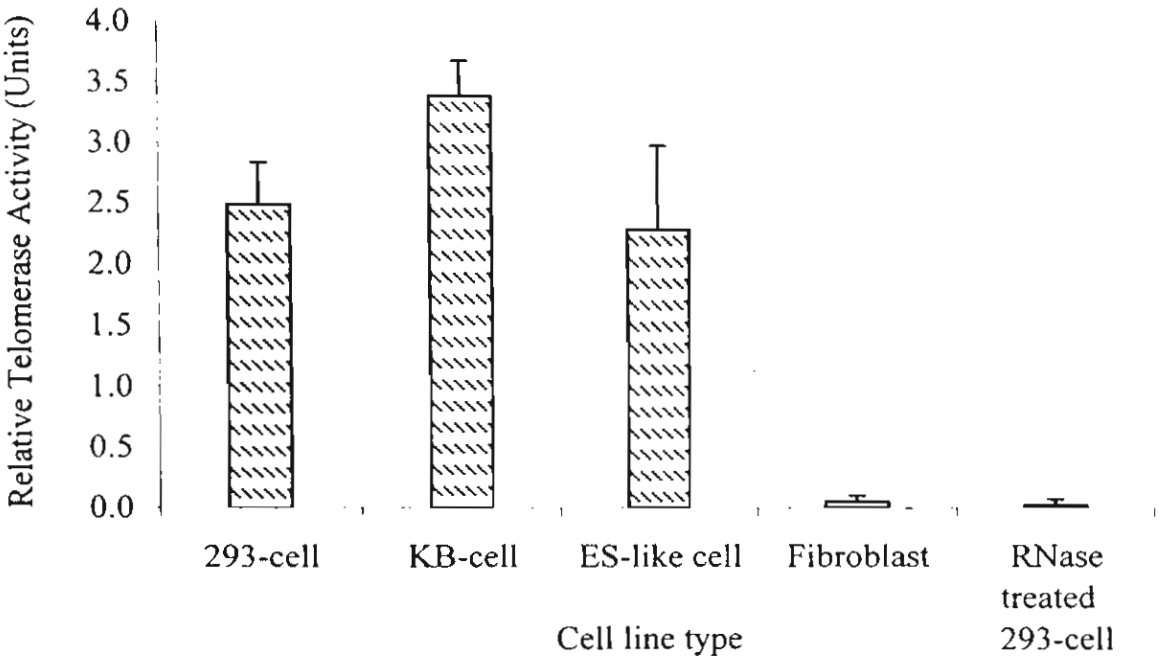

Fig. 1. Relative telomerase activity (RTA) in cell extracts of positive and negative cell lines. Cell extracts from 293-cell, KB-cell, buffalo ES-like cell, buffalo fetal fibroblast, and RNase treated 293-cell were diluted to 100 cell equivalents. Data are the means ± SEM of five replications.

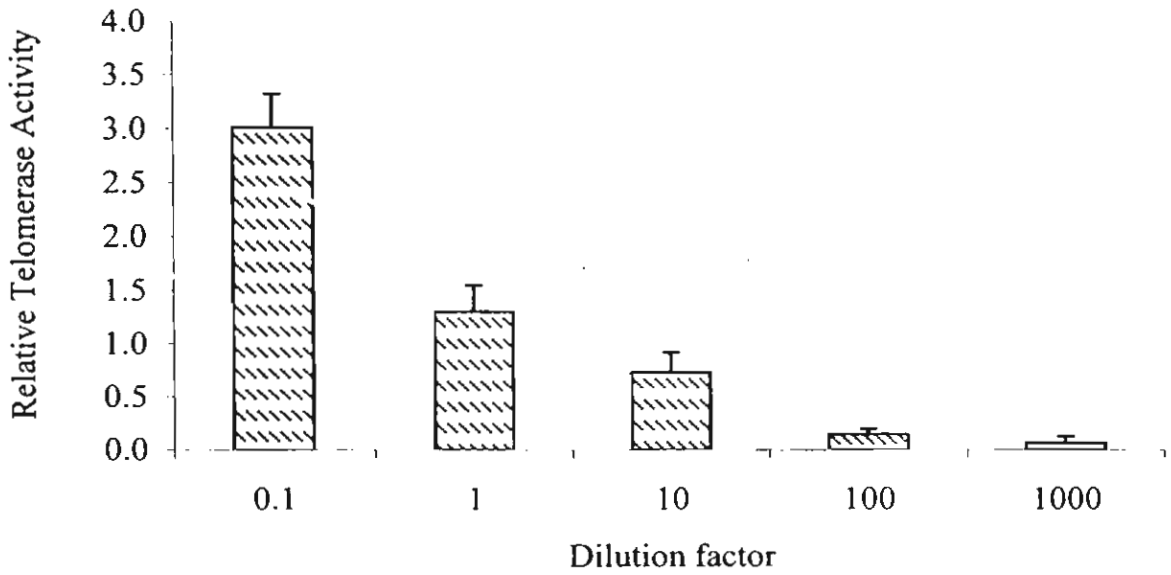

Fig. 2. Relationship between relative telomerase activity (RTA) and telomerase positive 293-cell extract. RTA was measured in 10-fold dilutions of cell extracts using the the Telomerase PCR ELISA kit and absorbance was measured at 450 nm to represent RTA of the corresponding cell dilutions. A linear relationship is found between RTA and dilution factor of the cell extract ($r = 0.93$).

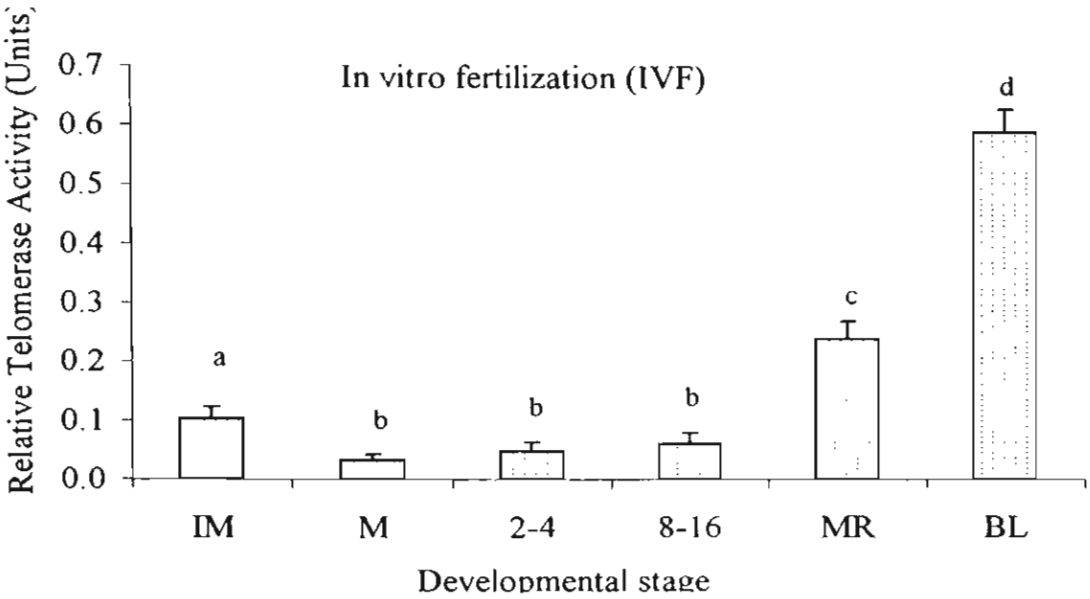

Fig. 3. Relative telomerase activity (RTA) in buffalo oocytes and IVF embryos. RTA was measured in 1 mg protein extracts from immature oocytes (IM), mature oocytes (M), 2-4-cell embryos (2-4), 8-16-cell embryos (8-16), morula (MR), and blastocyst (BL). RTA decreased from immature to mature oocytes and increased significantly at the morula stage. Highest level of telomerase activity was detected in the blastocyst stage. Different letters above the bars represent significant differences ($P < 0.05$).

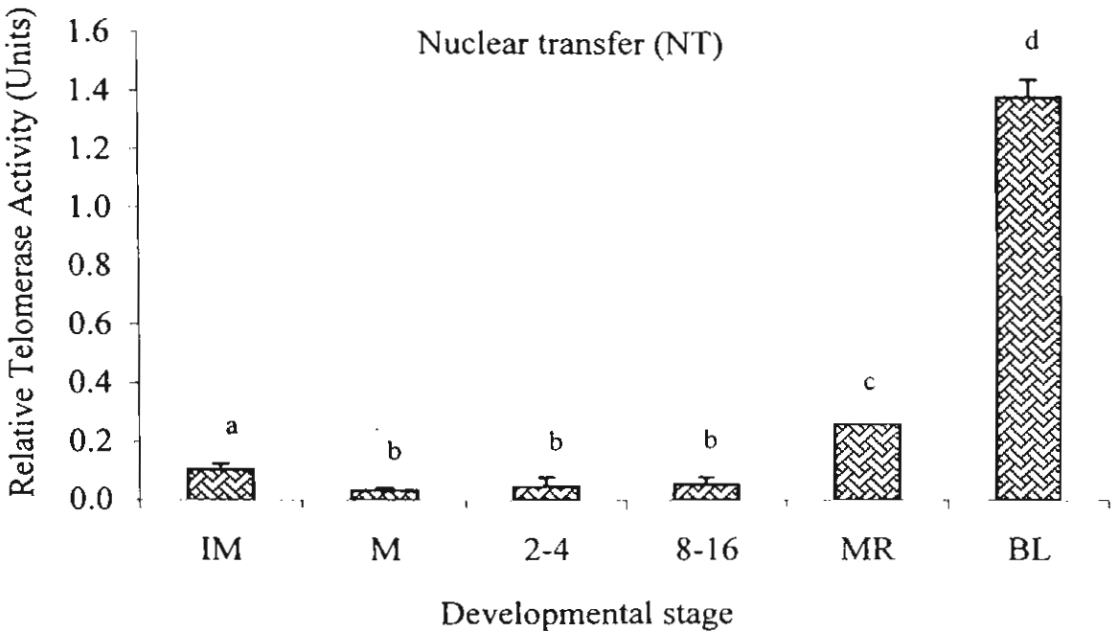

Fig. 4. Relative telomerase activity (RTA) in buffalo oocytes and NT embryos. RTA was measure in 1 mg protein extracts from immature oocytes (IM), mature oocytes (M), 2-4-cell embryos (2-4), 8-16-cell embryos (8-16), morula (MR), and blastocyst (BL). RTA decreased significantly from immature to mature oocytes ($P < 0.05$). A significant increase of telomerase activity was found in the morula stage and reached highest level at the blastocyst stage. Different letters above the bars represent significant differences ($P < 0.05$).

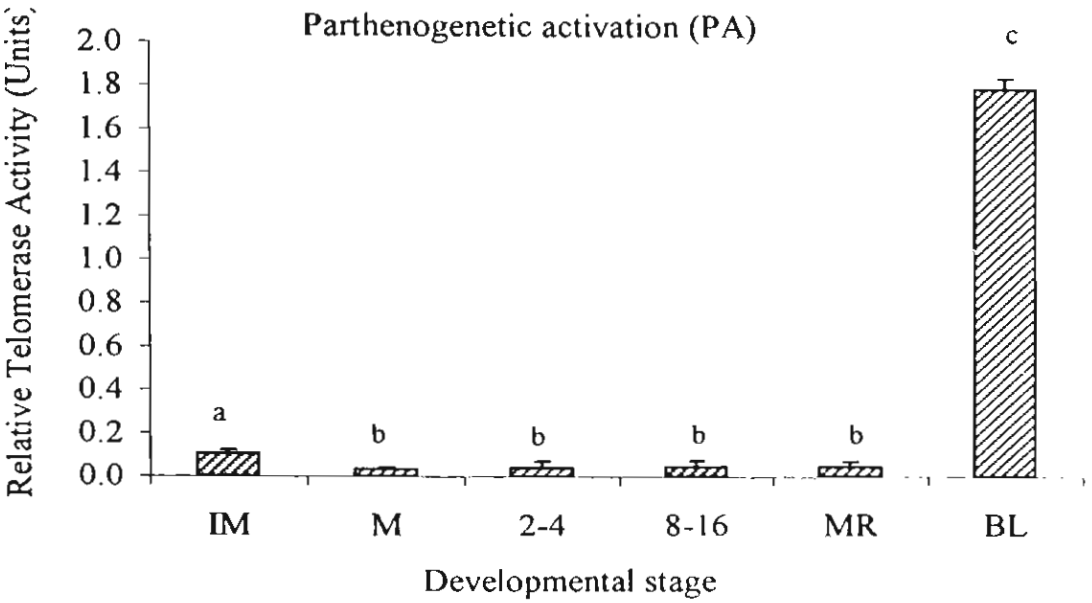

Fig. 5. Relative telomerase activity (RTA) in buffalo oocytes and PA embryos. RTA was measured in 1 mg protein extracts from immature oocytes (IM), mature oocytes (M), 2-4-cell embryos (2-4), 8-16-cell embryos (8-16), morula (MR), and blastocyst (BL). RTA decreased during oocyte maturation and had low level until the morula stage. A significant increase of telomerase activity was found in blastocyst stage embryo. Different letters above the bars represent significant differences ($P < 0.05$).

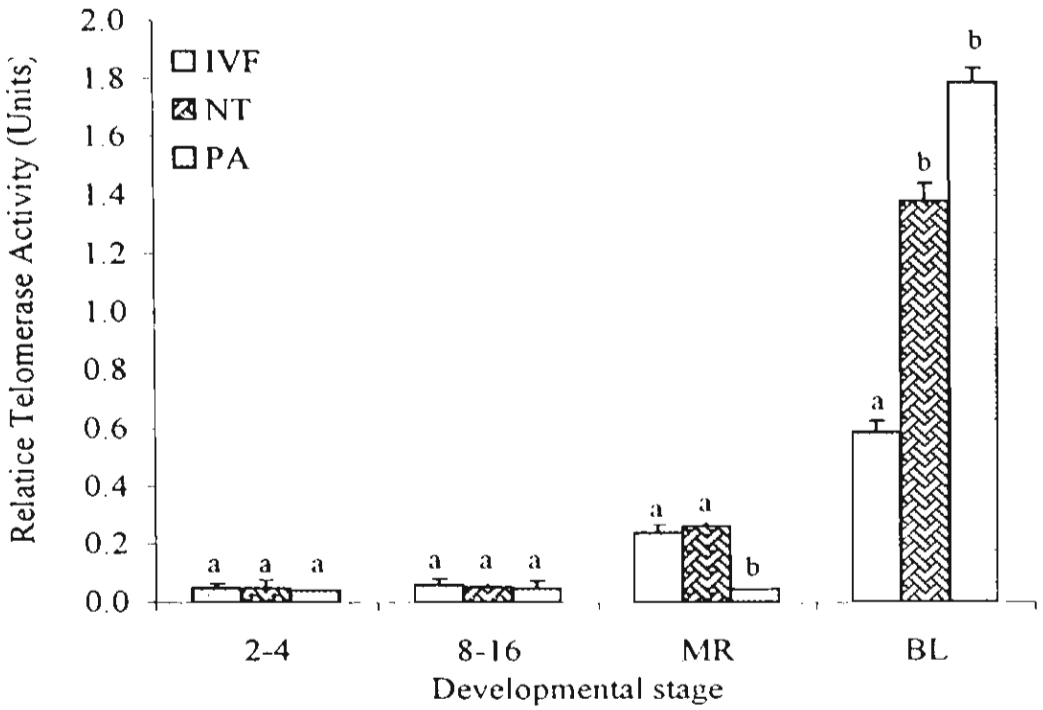

Fig. 6. A comparison of relative telomerase activity (RTA) in preimplantation stage embryos derived from IVF, NT, and PA. Blastocyst stage embryos have higher telomerase activity than other stages in all three systems. The telomerase activity in blastocyst stage embryos of PA and NT systems are higher than that of such embryos in the IVF ($P < 0.05$). Columns with different superscripts within each stage are significantly different ($P < 0.05$).