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EFFECT OF CULTURE SYSTEM ON THE SURVIVAL OF CRYOPRESERVED BOVINE EMBRYOS

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SUMMARY

This study compares the effect of culture system on the survival and hatching rates of in-vitro produced bovine embryos cryopreserved by slow freezing and vitrification. Oocytes were collected by slicing of ovaries from slaughtered cows. Presumptive zygotes produced by IVM/IVF were randomly allocated to one of the following culture media; TCM-199 or SOF. Day 7 and day 8 blastocysts, cultured in TCM-199 or SOF, were subjected to two cryopreservation methods; slow freezing and vitrification. Development of blastocysts 4, 24 and 48 hours post-thawing and hatching rates were recorded. There were no statistical differences in the post-thawing development or hatching rate between day 7 and day 8 cryopreserved blastocysts cultured in TCM-199 or SOF. But a significant difference was observed in the post-thawing development (4, 24, and 48 hours) of day 7 vitrified blastocysts cultured in SOF (46.1, 67.4, and 70.7%, respectively), as their survival rates were better than those cultured TCM-199. Day 7 and day 8 blastocysts culture in SOF had a better post-thawing development and 48 hours) after slow freezing than those cultured in TCM-199. Slow frozen and vitrified blastocysts cultured in SOF hatched more than the slow frozen ones cultured in TCM-199. In summary, day 7 vitrified blastocysts cultured in SOF were more cryotolerant. Cryopreserved blastocysts that were cultured in SOF survived vitrification and slow freezing better than those cultured in TCM-199.

INTRODUCTION

The establishment of in-vitro fertilization and culture systems for mammalian embryos has facilitated the application of embryo technologies in research, industry, and clinical applications. The quality of in-vitro produced blastocysts continually lags behind that of blastocysts produced in vivo (Boni et.al. 1999; Viuff et.al.1999; Corco-

ran et.al. 2006). In-vitro culture systems may result in embryos of reduced morphological quality compared with those produced in vivo (Rizos et.al. 2003). It had been demonstrated that the culture medium influence the mRNA expression (Wrenzycki et.al. 2001). The period of post fertilization culture is the period having the greatest impact on blastocyst quality as several major developmental events take place. These include the first cleavage division, the timing of which is known to be an important indicator of the subsequent developmental potential of the embryo (Lonergan et.al. 1999); the switching on of the embryonic genome (Memili and First, 2000); compaction of the morula, which involves the establishment of the first intimate cell to cell contacts in the embryo and blastocyst formation. Any modifications of the culture system, which could affect any or all of these processes, could have a major effect on the quality of the embryo (Rizos et.al. 2001). The end point conventionally used to measure the efficacy of a culture system for in-vitro embryo studies is the percentage of blastocysts formation, and it has become routine to select blastocysts following a standard culture period for cryopreservation or embryo transfer (Kubisch et al. 2001). Furthermore, the faster developing blastocysts in in-vitro culture systems are generally considered more viable and better able to survive following cryopreservation or embryo transfer than those that develop more slowly (Dinnyes et.al. 1999).

Cryopreservation of embryos has become an important procedure in assisted reproduction technology. It provides the possibility of preserving female fertility in reproductive medicine as a therapeutic strategy. The technology applied to cryopreserve embryos is well documented. The slow-freezing/rapid-thawing method appears to be effective for the embryos of various mammalian species including humans (Huang, 2007). Recently, vitrification technology has become an attractive alternative to slow-freezing because it appears that vitrification results in significantly higher survival and pregnancy rates (Kuleshova and Lopata, 2002).

In-vitro-produced (IVP) bovine embryos are characterized by an increased chilling sensitivity and a lower freezability compared to their in vivo counterparts (Pollard and Leibo, 1993). Loss of viability after cryopreservation is probably due to cellular damage or metabolic disturbances occurring during the procedure. The type and degree of such cryoinjuries are likely to depend on the method of cryopreservation (Kaidi et.al. 2001). Advances in embryo survival following cryopreservation could be achieved by improving their culture conditions. Culture system can affect blastocyst yield and quality, and crytolerance is a useful indicator of blastocyst quality (Rizos et.al. 2001).

This work aimed to study the effect of two different culture systems on the survival rate of in-vitro produced bovine embryos (day 7 vs. day 8 blasto-,

cysts) as assessed by cryopreservation either by slow freezing or vitrification.

MATERIAL AND METHODS

All chemicals were purchased from Sigma Chemical Co. (Sigma-Aldrich Chemie Gmbh, Munich, Germany).

Oocyte collection and in-vitro maturation (IVM)
Bovine ovaries were collected from a local abattoir (Munich, Germany) and transported to the laboratory (within 2h) in sterile 0.9% NaCl solution at 35-38°C. Cumulusñoocyte complexes (COCs) were obtained by slicing 2- to 10-mm follicles (in slicing media, modified PBS) on the surface of the ovaries obtained from slaughtered cows (Paula-Lopes and Hansen, 2002). Only COCs that had at least three layers of compact and unexpanded cumulus cells were selected under a stereomicroscope. The COCs were washed three times and then cultured in TCM-199 supplemented with 50 mg/mL gentamycin, 5.5 mM Ca lactate, 2.2 mM Na pyruvate, 36 mM NaHCO3, 5 mM Hepes, 0.01 UI/mL FSH and 10% estrous cow serum (ECS). Maturation was performed in four-well plates (Nunc, Denmark) in groups of 30 COCs in 500 mL of maturation media for 22ñ24 h at 38.5∞C under 5% CO2 in air with maximum humidity (Minitube, Tiefenbach, Germany).

In-vitro fertilization (IVF)
Motile spermatozoa were obtained by centrifugation of frozen-thawed bull semen. Matured COCs were transferred to new four-well plates containing 500 mL of Tyrodes solution with albumin, lactate and pyruvate (TALP), supplemented with 50 mg/mL heparin. Spermatozoa were counted and an aliquot of sperm suspension was added to each well, to obtain a final concentration of $1x10^6$ spermatozoa/mL. Plates were incubated for 24 h at 38.5°C under 5% CO_2 in air with maximum humidity (95%).

In-vitro culture (IVC)
At 20 hours post insemination (hpi), presumptive zygotes produced by IVM/IVF were randomly allocated to one of the following culture media: TCM-l99, cultured in 5% CO2 in air, n=978; or SOF, cultured in 5% CO2, 5% O2, and 90% N2, n=713. The cleavage rate was recorded at 72 hpi. Blastocyst production was recorded on Day 7 and 8 post-insemination.

The effect of post-fertilization culture medium on the proportion of bovine zygotes developing to the blastocyst stage and on the quality of the blastocysts produced was assessed by survival after cryopreservation. Day 7 and 8 blastocysts from each culture medium were subjected to either verification/warming or slow freezing/thawing.

Vitrification and warming
Blastocysts were vitrified using the minimum volume cooling (MVC) method (Kuwayama and Kato, 2000). Embryos were equilibrated with

7.5% ethylene glycol (EG), 7.5% dimethyl sulfoxide (DMSO), and 20% fetal calf serum (FCS) and then exposed to 15% EG, 15% DMSO, 0.5 mol/l sucrose and 20% FCS for 30 sec. Embryos were loaded onto a MVC strip (Cryotop; Kitazato Supply, Tokyo, Japan)) and plunged into liquid nitrogen. For warming, embryos were recovered into a 0.25 mol/l sucrose solution for 1 min, transferred into 0.15 mol/l sucrose for 5 min, and cultured in TCM + 10% FCS.

Slow freezing and thawing

The freezing procedure was that described by Massip and Van Der Zwalmen (1984). EG and sucrose were used as cryoprotectants. Blastocysts were pooled in embryo transfer freezing medium (ETF). They were exposed for 10 min at room temperature to a mixture of 1.5 M EG and 0.25 mol/l sucrose in ETF supplemented with 20% FCS. Groups of 3 embryos were loaded in this mixture into 0.25-ml straws (L'Aigle, France) between two columns of 0.5 mol/l sucrose in ETF separated by air bubbles. The straws were placed vertically into a freeze control machine (HAAKE, Phoenix II, Germany) and pre-cooled at -7.5°C for 10 min (including seeding). Straws were then cooled at 0.2°

C/min down to -30°C, following which they were immersed and stored in liquid nitrogen. For thawing, the straws immersed for 10 sec in a water bath at 38°C. The contents of each straw were emptied in a Petri dish and mixed by slight agitation. Embryos were transferred to ETF medium for 5 min at room temperature and then cultured in TCM + 10% FCS.

Post-cryopreservation survival rate

After thawing or warming, embryos were recovered and washed in culture medium (TCM-199 + 10% FCS) and then cultured in 50µl drops of this media in a humidity atmosphere of 5% CO_2 in air. Re-expansion and hatching rates were defined as the percentage of blastocysts that had respectively re-expanded and hatched over the total number of blastocysts. Re-expanded and hatched embryos were evaluated at 6, 24 and 48 h post-thawing/warming.

Treatment effects on cleavage rate, blastocyst yield, survival rate after thawing/warming and blastocyst hatching rate were determined by ANOVA models (Noursis, 1986).

RESULTS

Blastocysts, day 7 and day 8, that were cultured in TCM-199 or SOF were cryopreserved either by vitrification or slow freezing and their viability was evaluated post-thawing at 4, 24, and 48 hours. Additionally, the percentage of all hatched blastocysts cultured in each medium was recorded.

In TCM-199, development means of vitrified blastocysts after 4, 24, and 48 hours post-thawing

were higher (P<0.05) than those cryopreserved by slow freezing. Also, in SOF, percentages of post-thawing development of vitrified blastocysts at 4 and 24 hours were higher (P<0.05) than those cryopreserved by slow freezing (Table 1, Fig. 1). Furthermore, the post-thawing development of blastocysts cultured in SOF was higher than those cultured in TCM-199 after both vitrification and slow freezing. Also, blastocysts that were cultured in SOF hatched more than those cultured in TCM-199 (Table 1).

There was no statistical difference in the post-thawing development between day 7 and day 8 vitrified or frozen blastocysts cultured in TCM-199 or SOF. While, there was a significant difference (P<0.05) between day 7 vitrified blastocysts cultured in SOF compared with TCM-199 (Table 2, Fig. 2). There was no statistical difference in percentage of hatched blastocysts vitrified and frozen, and cultured in TCM-199 or SOF. Slow freezing of day 7 and day 8 blastocysts, cultured in TCM-199 or SOF, have shown significant differences (P<0.05) in their development after 4 and 48 hours post-thawing (Table 2, Fig. 3).

Table 1: Survival rate of vitrified and frozen bovine blastocysts cultured in TCM-199 or SOF

IVC Media	Blastocysts (n)	Post-thawing development (%)			
		4 h	24 h	48 h	Hatched
TCM					
Vitrification	79	21.7±5.4 ^{a}	42.1±6.5 ^{a}	42.1±6.5 ^{a}	7.6±2.3 ^{ab}
Slow freezing	92	5.8±2.3 ^{b}	19.1±2.5 ^{b}	24.5±2.3 ^{b}	5.5±2.4 ^{ac}
SOF					
Vitrification	47	47.1±3.5 ^{c}	65.6±7.5 ^{c}	68.1±6.1 ^{c}	13.0±5.1 ^{b}
Slow freezing	95	24.7±4.0 ^{a}	47.1±2.4 ^{a}	53.5±2.8 ^{ac}	13.8±2.3 ^{b}

Means (±SE) with different superscripts ($^{a-c}$) in the same column are significantly different (P<0.05).

Table 2: Survival rate of day 7 and day 8 bovine blastocysts cultured in TCM-199 or SOF and cryopreserved by vitrification or slow freezing

IVC Media	Blastocysts (n)	Post-thawing development (%)			
		4 h	24 h	48 h	Hatched
Vitrification:					
TCM: Day 7	37	14.1±8.7 ^{a}	32.5±9.3 ^{a}	32.5±9.3 ^{a}	5.8±3.6 ^{a}
Day 8	42	28.1±6.2 ^{a}	50.0±8.3 ^{a}	50.0±8.3 ^{a}	9.0±3.1 ^{a}
SOF: Day 7	26	46.1±4.7 ^{b}	67.4±10.3 ^{b}	70.7±7.7 ^{b}	14.1±7.1 ^{a}
Day 8	21	47.1±3.5 ^{ab}	65.5±7.5 ^{ab}	68.1±6.1 ^{ab}	13.0±5.1 ^{a}
Slow freezing:					
TCM: Day 7	35	2.8±2.7 ^{a}	16.9±5.2 ^{a}	22.7±5.0 ^{a}	5.6±5.5 ^{a}
Day 8	57	8.9±2.5 ^{a}	21.2±1.8 ^{a}	26.3±0.5 ^{a}	5.4±2.2 ^{a}
SOF: Day 7	53	24.5±2.3 ^{b}	45.3±4.6 ^{a}	54.7±2.9 ^{b}	17.0±2.2 ^{a}
Day 8	42	24.9±9.5 ^{b}	48.9±2.7 ^{a}	52.3±6.2 ^{b}	10.7±3.0 ^{a}

Means (±SE) with different superscripts ($^{a-b}$) in the same column are significantly different (P<0.05).

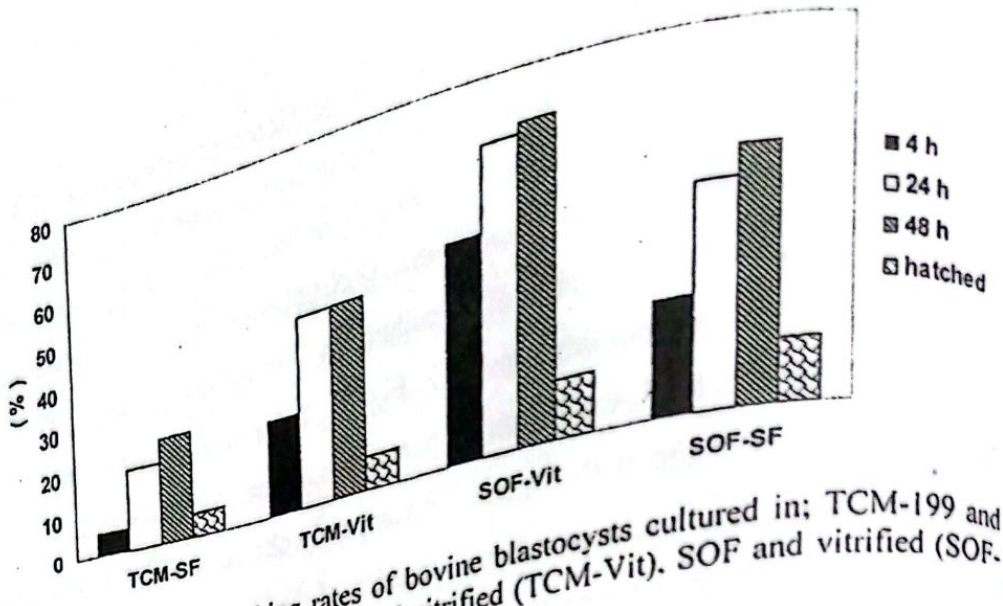

Fig 1: Survival and hatching rates of bovine blastocysts cultured in; TCM-199 and slow frozen (TCM-SF), TCM-199 and vitrified (TCM-Vit). SOF and vitrified (SOF-Vit), and in SOF and slow frozen (SOF-SF).

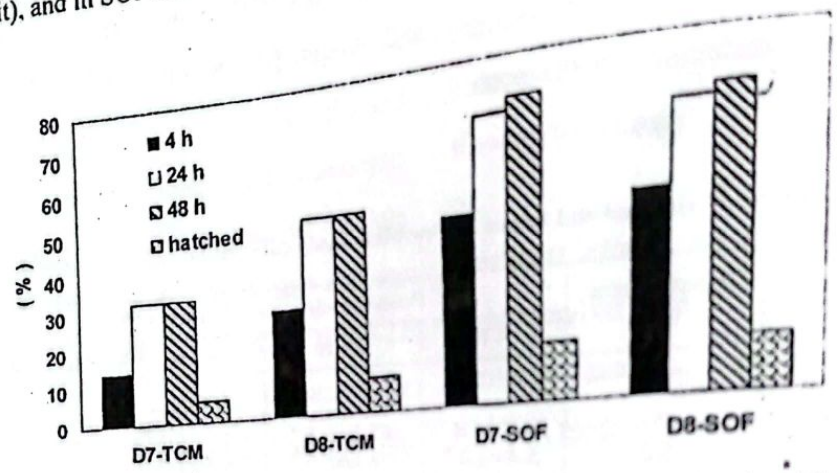

Fig 2: Survival and hatching rates of day 7 (D7) and day 8 (D8) bovine blastocysts cultured in TCM-199 or SOF and cryopreserved by vitrification

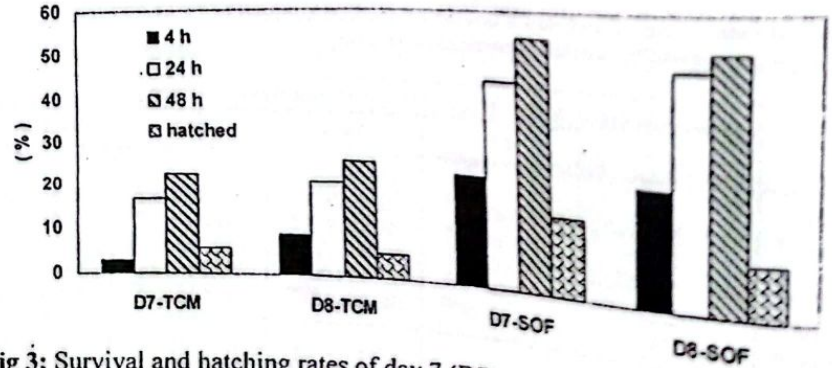

Fig 3: Survival and hatching rates of day 7 (D7) and day 8 (D8) bovine blastocysts cultured in TCM-199 or SOF and cryopreserved by slow freezing

DISCUSSION

Cryopreservation of embryos has become a wide-ly used method in commercial embryo trans-fer because survival rate of cryopreserved/thawed embryos is nearly comparable with fresh embryos (Shaw et al., 2000). Nevertheless conven-tional cryopreservation (slow freezing) is a slow proce-dure which exposes the embryo at various phases of freezing to the action of many physical, chemi-cal and biologi-cal factors. These factors can cause disruption of the zona pellucida, cell mem-branes or cytoskeleton and metabolic disturbanc-es. Such cell damage leads to loss of self-control of the cell and eventually to its death by apopto-sis or necrosis (Baguisi et al., 1999). Formation of intracellular ice crystals is con-sidered the most harmful factor which can occur under spe-cific conditions of freezing and thawing with neg-ative effect on recuperation and survival of em-bryonic cells (Vajta, 2003; Vajta and Kuwayama, 2006). Vitrification, as an alternative method of cryopreservation, uses a much more concentrated cryo-protectant and its high cooling rate (20 000-25 000 or more°C/min) prevents the formation of crystal-line ice. Instead, the high viscosity of the cryopro-tectant forms a solid glass-like mass. Vit-rification considerably simplifies and accelerates the cryop-reservation process without requiring expensive equipment (Celestinos and Gatica, 2002; Vajta, 2003). This method is also useful for cryopreservation of in-vitro matured bovine oo-cytes (Papis et al., 2000; Asada et al., 2002).

Survival and hatching rates were compared, in this study, between in-vitro produced bovine blastocysts cultured in TCM-199 or SOF and cryopreserved by slow freezing and vitrification. No statistical differences were observed in the method of cryopreservation of blastocysts cul-tured in TCM-199, although blastocysts survived vitrification process more than frozen blastocysts. Blastocysts cultured in SOF survived vitrification better than frozen blastocysts, after 4 and 24 hours post-thawing. Furthermore, blastocysts cul-tured in SOF survived vitrification and slow freezing better than blastocysts cultured in TCM-199. Nandi et.al., (2003) have shown that the mean percent of morphologically normal blasto-cysts after thawing and their survivability were significantly higher in blastocysts obtained from embryos cultured in SOF than those cultured in TCM. Kaidi et.al., (2001) have reported that im-mediately after thawing, the proportion of em-bryo cells showing membrane alterations and a decrease in total cell number were similar in fro-zen and vitrified bovine embryos (15-17%). These membrane alterations observed after freez-ing are likely to be due to ice crystal formation or osmotic shock. They added that after culture in SOF, survival and hatching rates were decreased in both frozen and vitrified embryos by compari-son with untreated ones. This decrease could be due to a slowing in embryonic cell proliferation after cryopreservation. Frozen blastocysts had fewer cells than vitrified ones after several days of culture. This seems related mainly to a more

marked decrease in trophectoderm cells after freezing, while the decrease is similar in both cell lineages in vitrified embryos (20 and 24% for trophectoderm and inner cell mass, respectively). The vitrification procedure may cause damage to blastocyst cells resulting in an increase in DNA fragmentation and apoptosis-related gene transcription, reducing developmental capacity of vitrified bovine blastocysts (Park et al., 2006). On the other hand, Huang et al. (2007) have reported that there was no difference in the survival and hatching rate of the bovine blastocysts between control (62.5%) and vitrified (61.7%) groups, and the number of dead cells in the blastocysts was not significantly different between control and vitrified groups. De Rosa et.al. (2007), have demonstrated that cryotop vitrification is a valid tool to cryopreserve IVP buffalo blastocysts yielding a high embryo survival rate (67.9%).

In this study, differences were observed in the post-thawing development of cryopreserved day 7 and day 8 blastocysts cultured in SOF, as their survival rates were better than those cultured in TCM-199 (Table 2). Nedambale et.al., (2004) have reported that day 7 vitrified blastocysts cultured in SOF had higher development at 6 (71%), 24 (64%), and 48 hours (60%) post-warming compared to slow freezing (48, 40, and 31%, respectively). In another study by Nedambale et.al., (2004), they demonstrated that embryos that cleave and reach the blastocyst stage earlier survive cryopreservation better than those that take a longer time in development. Mucci et.al., (20 have reported that vitrification yielded the high post-cryopreservation survival rates compared slow freezing regardless the presence of serum the culture medium.

In conclusion, the cryopreserved blastocysts th were cultured in SOF survived vitrification a slow freezing more than those cultured in TC 199. Slow frozen blastocysts cultured in SC hatched more than those cultured in TCM-19 The results of the present study indicate that b vine embryos show temporal sensitivity to t culture environment after cryopreservatio which is manifested in terms of the quality of t blastocysts produced.

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تأثير انظمة المنابت على بقاء اجنة الابقار المجمدة

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تقارن هذه الدراسة تأثير انظمة المنابت على نسب الفقس و البقاء لاجنة الابقار المنتجة معمليا و التى تم تجميدها بطريقتى التجميد البطيئ و التزجيج. تم تجميع البويضات بتقطيع المبايض من الابقار المذبوحة. تم توزيع الاجنة الافتراضية المنضجة والملقحة معمليا بشكل عشوائى على واحدة من المنابت الاتية: SOF , TCM-199. ثم تم تجميد الاجنة عمر 7 ايام و 8 ايام بطريقتى التجميد البطيئ و التزجيج. سجلت نسب الفقس و البقاء للاجنة بعد 4 ، 24 ، 48 ساعة من عملية الاذابة. لم تكن هناك فروق معنوية فى نسب الفقس و البقاء بين عمر 7 ايام و عمر 8 ايام للاجنة المجمدة. ولكن لوحظ وجود فروق معنوية فى تطور الاجنة بعد الاذابة (4 ، 24 ، 48 ساعة) وذلك فى الاجنة عمر 7 ايام والتى تم انباتها فى SOF وتجميدها بطريقة التزجيج ، حيث كانت نسب البقاء (46.1 ، 67.4 ، 70.7% ، على التوالى) افضل من التى تم انباتها فى TCM-199. أيضا كانت الاجنة التى تم انباتها فى SOF وتجميدها بطريقة التجميد البطيئ ، تطورها افضل بعد الاذابة (4 ، 48 ساعة) من التى تم انباتها فى TCM-199. كما ان الاجنة التى تم انباتها فى SOF وتجميدها سواء بالتجميد البطيئ او بالتزجيج كانوا افضل تطورا من هؤلاء الذى تم انباتهم فى TCM-199 وتجميدهم بطيئا. فى الخلاصة، يمكن القول ان الاجنة عمر 7 ايام التى تم انباتها فى SOF ، كانت اكثر مقاومة للاثار السلبية لعملية التجميد. ايضا الاجنة التى تم انباتها فى SOF والتى تم تجميدها بطريقتى التجميد البطيئ و التزجيج ، كانوا افضل من حيث التطور عن هؤلاء الذى تم انباتهم فى TCM-199.