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In Vitroenergy Requirements of in Vitro and in Vivo Derived Embryos of Cross Bred Cows Synchronized and Super Ovulated with Crestar and Porcine Follicle Stimulating Hormone

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Abstract **–The considerable progress made during the last decade in the development of the synchronization and superovulatory responses in animals and this worked was plane to study on the cross bred cows and the characterization of energy requirements of the pre implantation embryos from the 8- cell embryos onward after being produced invitro and invivo. Animals were kept in different private farms under strict supervision of the JahadKeshavarzi and government veterinary section in the Damghan city, Semnan province in Iran as a joint work carried out by the Zabol veterinary college and Haryana Agricultural college , India . Cross bred cowsin winter (n=12) and summer (n=12) seasons between 2-5 years of aged were assigned randomly to receive a total dose of 75 International Unites of Super-ov(FSH-P)for 3 days in a descending -orders from Day -2 through Day 0 (-day of implant removal) to induce superovulation . Estrus synchronization was done by insertion of a 3 mg synthetic progestogene (Crestar) on the external surface of the ear for 14 days. In the middle of heat, after first mating with fertile bull, each cow was injected with 2.5 mL of receptal(Gn-RH) to ensure that the cows ovulated . Non invading embryo collection wasdone on Days 6 and 8 post estrus (Day0) and collected embryos were subjected to comparison of energy requirement by the same stage of in-vitro derived embryos. A majority of cross bred cows (19/24) exhibited estrus within 8 hr after implant removal . Theeffect of the seasons on the CL numbers, un-ovulated follicles and overall ovarian activity, estrus behavior, super ovulation and embryos quality for summer and winter seasons were recorded. While the pattern of energy requirement from the 12 -cell stage onward was constant among invitro and invivo derived embryos, lower ratio ofpyruvate substratewas preferred by the embryos in vitro. Lactate consumption reached up to 50% of pyruvate level,except at8- cell and early morula stages which required glucose, and glucose concentration has steady increased up to blastocysts stages.Oxidation and CO2 production of lactate invitro was significantly higher as compared toin vivo. Low viability of collected embryos at the morula and blastocysts stages was due to the thawing of freeze sperm and fertilization invitro and after embryo transfer into the receiver uterus, but level of CO2 production from glucose after freezing process in in vitro remained unchanged.**

Keywords **– Ear Implant, Energy Requirement, Substrates, Super-Ov.**

I. Introduction

A major technical breakthrough was achieved during the last decades as a system for generating bovine embryos through Synchronization of estrus and superovulationas a basic techniques for embryo transfer programs. Reports of Super stimulation of Angus donors with a single intramuscular injection of Folltropin-V.[1,2]. Following researches indicated that the reproductive performance of dairy cattle is affected by seasonal variation [3,4], the summer season slow done the follicular growth[5], decrease CLactivity [6]depress estrus signs[7], modify super ovulatory response [8], decrease quality of embryos[9,10], and oocystfertility in cattle[11].

The metabolic rate of an embryo reflects its capacity to carry out functions necessary for its well being and may serve as a suitable index to investigate embryos health. [12], [13] observed no difference in superovulatory response and in total corpora lutea produced when diets are taken into consideration.

However [14] stated that, a proper increase in the diet's energetic level may increase the number of follicles that respond to the superovulatory diet. Moreover, the dietary source used to feed the donors may also influence their superovulatory response and the quality of their embryos [15], [16]. Recent research highlights the benefits with sources rich in essential fatty acids in the diet. [17] observed that the number of viable embryos increased when grains of canola (linoleic acid n-6) were added to the diets of Nellore heifers. An increase in the energy density of the diet may increase the number of responsive follicles [18]. [17] observed that heifers fed on canola seed presented a higher number of corpora lutea in the left ovary, a lower number of cysts in the right ovary and fewer total cysts. These results were similar to those observed by [16]in their research on Holstein cows that received linseed grains or Megalac®. The amount of fat in the diets of cows increases the number of follicles, although this increase occurs regardless of fatty acid profile [16].

Measured glucose utilization and lactate production of day 10 cow embryos and found that the group of embryos with a higher glucose consumption was more competent of further development when transfer to suitable recipients or placed in culture. While glucose is the major source of metabolic energy for all mammalian tissues, the early stages of mammalian development can not be supported by this substrate. Marked increases in the glucose requirement can occur in cattle embryos between the 2-cell and the blastocyst stages. [19],[20].

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The production of carbon dioxide from glucose has been measured in bovine by (Rieger and Guay) and [19],[20],[21],[22] embryos and large differences among the species have been fouynd. In general ,the amount of CO_2 production ,is low until the 8-cell stage and then rises at all stages to the blastocyst. There are 2 known path ways used by the embryos for CO_2 production from glucose. Little glucose seems to be utilized through TCA cycle by the embryos of domestic species [21] , they stated that in bovine blastocyst the TCA cycle was active but glycolysis was blocked either due to the absence or inhibition of Pyruvate kinase. [22] studied the effects of sex and stage of development on the activities of different energy consumption path ways in individual day 7 cow embryos. Total glucose consume by male embryos was two times more than that of female embryos and increased between then morula and expended blastocyst stage. Some investigators reported a detrimental effect of glucose on embryonic development in vitro in a number of species including cattle[23].In another study , a higher proportion of in- vitro derived bovine zygotes formed blastocysts when phosphate was reduced to 0.5 Mm and glucose was added to the medium after 72 hr of culture[23].

The objective of this experiment was to evaluate the synchronization and superovulatory response to Crestar and FSH-P and to compareenergy requirement of*invitro* and *invivo*derived embryos from cross bred cows with *invitro*produced embryos of the same stage from the 8-cell stages onward using methods to study *invitro*development to enhance. Understanding of energy requirement of pre implantation embryos with respect to improving the yield and quality of embryos.

II. Materials

The present study was conducted with 24cross bred cows, 2-5 years of age selected on random basis. The animals were kept under intensive international health care. Cows and bulls were kept in separate places and mix at the time of mating. After de- worming, cows were assigned to either winterseason (n=12 cows) or summer season (n=12 cows).

Estrus Synchronization and Super-Ovulation Protocols

1- Insertion of 3mg synthetic progestogene, norgestomet ear implant for 14 days.

2- 75 IU of FSH-P (Super Ov; Ausa International ,USA) was administered to each cow according to the following schedule.

Day -2: 2×16 IU at 12 hrinterval

Day -1:2×12 IU at 12 hr interval

Day 0:2×9. 5 IU at 12 hr interval

3- Before the last injection of FSH-P, ear implant were removed.

4- Eight hours after implant removal, cows on estrus were detected three times a day by teaser bulls as well as by persons familiar with the estrus signs.

5- Upondetection of estrous, cows were allowed mating naturally with potent healthy fertile bulls.

6- Cows administered 2.5 mL of synthetic Gn-RH analogue (Receptal) containing 0.01 mg of Buserelin one hr after 1st mating to ensure that the cows ovulated to improve conception rate .

Non invadingflushingmethod of embryos

All cows were flushed non surgically by using a silicone two way Foley Catheter (25 in 20: AB Technology, USA and its tube set (66 in; ab Technology, USA) to allows flushed oviducts and uterine horns contents on Day6 or Day 8 on each sub groups (n=6) for winter and summer seasons) using Dulbecco's Phosphate Buffer Saline. (ZT156; IMV, France) flushing medium. In general the ovarian response of all cows were recorded as described previously by [24].

The procedure followed for estimating energy requirement of embryos and blastocysts is using Assay of radioactivity for CO_2 and Lactate productions

Embryo metabolism

The data obtained for CO_2 production and substrate carbon uptake from all three energy substrates by the embryos collected in vivo and matured in vitro or collected and matured in vitro are given in tables 3 and 4th .While the pattern of oxidation was similar between embryos from the two sources, Pyruvate were preferred substrate by both the sources. No significant differences were observed in oxidation of glucose between the two sources of embryos or the two concentrations of substrate. In general, the energy consumption remained unchanged as the 2-cell embryo advanced to the 8-cell stage. However, uptake of glucose carbon was 4 times greater in the 8-cell embryos than in the 2 cell embryos. The 2 concentrations of glucose did not differ significantly in their rate of energy consumption. Apart from the higher oxidation of lactate by the in vivo collected embryos of both stages, the energy consumption capacity of embryos obtained from the two sources remained similar.

Catabolic metabolism was evaluated by measuring the production of CO_2 from all three substrates and in addition lactate production from glucose. Anabolic metabolism was estimated by determining the amount of substrate carbon incorporated by the embryos.

A specially washed and heat sterilized 20 mLscintillation vial served as the incubation apparatus. Embryos were washed through two changes (2 mL) of energy-substrate- free PBS medium and placed in a 50µL drop of radioactive medium for a final wash before being transferred into a sterile, 600µL micro-centrifuge-tube (Eppendorf-Netheler-Hinz, Gmbh, Hamburg, Federal Republic of Germany) containing 50µL radioactive incubation medium.This tube was placed in the scintillation vial with the lid of the tube acting as a support.Simultaneously,0.5 mL of 2.5 mol/L sodium hydroxide was added on to the floor of the vial , which was then closed and incubated at 39°C for 4 hr. At the end of incubation, the tube with embryos was taken out and the radioactivity in the trapped CO_2 was determined after the addition of scintillation cocktail. In all experiments, at least two sham incubations were run concurrently to correct for background counts. The metabolic turnover was estimated from the disintegrations per minute detected in the sample and the specific activity of the parent substrate,and converted to picomoles/embryo/h on the

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basis that each glucose molecule gives rise to 6 molecules of CO_2[25].

Oocytes by a compact cumulus oophorous with about three layers were obtained and then by puncturing ovarian follicles of more than 10mm diameter with the help of needle no 24 gauge placed their content in the culture medium (DPBS), maturation medium (TCM-199 GibcoLab, New York, Earls salt, Centron Research Lab, India, and then was modified by the addition of sodium pyruvate22µg/ml, sodium bicarbonate2.2mg/mLand streptomycin sulphate 0.5mg/mLto prevent fungus formation.

Comparative metabolic studies of embryos

In the present study, the 8-cell stage embryo in vitro subjected to comparative energy consuming studies with in vivo derived embryos at the same stage of development for assessment of energy differences and requirement for further development up to hatched blastocyst stage.

The invitro and invivo and in vitro derived embryos was periodically assessed at every 4hr intervals.The energy consumption of 8 to16- cell stages onward to the hatched blastocyst was investigated by using 3 different substrates. Non invading recovered embryos at the stages of 8- cell to the morula and blastocyst stageswere subjected to comparative energy substrate consumption by the same stage embryos of in vitro.

Classification of recovered embryos

The classification of the recovered embryos were done according to International guidelines [18] after four times washed embryos in fresh medium ,the excellent and good quality embryos were used to studiesenergy requirement. These studies were carried out in four parts as follows

1- Eight-cell embryo were used to estimate energy requirement before block stages c prior to activation of the embryonic genome [26].

2- Morula stage embryos were used to elucidate changes toenergy requirementafter the block period and before the period of rapid growth.

3- Blastocyst stage embryos were classified as early, mid and expanded stage blastocyst before being used for energy estimation. The sizeof some of the embryos were measured with an ocular micrometer at two axes perpendicular to each other and was expressed as mean of the two measurements), measured before estimation of energy requirement to find relationships between embryosize and energy requirement. The invivoderived early blastocysts were cultured in vitro for 24 or 48 hr to obtain mid and expanded stage blastocysts respectively.

4- Hatching/hatched blastocysts were subjected to studies energy need to assess changes during the process of hatching and post hatching.

Statistical analyses

The statistical analysis of data for synchronization, super ovulation and embryo recovery were made by using methods described by [27].

III. RESULTS

In the present study, only 6cross bred cows exhibited estrus at 18 hr after implant removal).

Ovarian response of cross bred HF cows following superovulation during winter season was tabulated in (table 1). Ovarian response of cross bred HF cows following super ovulation during summer season was tabulated in (table 2). The comparative data during summer season inferred somewhat lower ovulation compared to winter season (tables I and II).

Means with different superscripts(a,b,c,d) in the each row are different(P < 0.005:

Table I: Ovarian response of cross bred cows following superovulation during the winter season

Number of animals			Corpora Lutea			Unovulated follicles			Overall Ovarian activity
FSH-P Treated	Right Ovary	Left Ovary	Total	Right Ovary	Left Ovary	Total	Right Ovary	Left Ovary	Total
12	49	67	116	14	7	21	63	74	137
Mean±se	4.08 ±0.60	5.59±0.84	9.67±1.18	1.17±0.49	0.58±0.36	1.75±0.66	5.25±0.90	6.17±0.94	11.25±1.66

Table II: Ovarian response of cross bred cows following superovulation during the summer season

Number of animals			Corpora Lutea			Unovulated follicles			Overall Ovarian activity
FSH-P Treated	Right Ovary	Left Ovary	Total	Right Ovary	Left Ovary	Total	Right Ovary	Left Ovary	Total
12	38	45	83	8	2	10	46	47	93
Mean±se	3.17±1.02	3.75±0.52	6.92±1.41	0.67±0.28	0.17±0.11	1.83±0.34	3.83±1.20	2.91±0.53	7.75±1.60

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Embryos flushing

Embryos Flushing on day 8 during winter and summer seasons yielded embryos with the developmental stages varying between 8- cell tomorula.The majority of embryos were between 16 cell stage and blastocyst ,two embryos (oneembryo 4- cell and another one 8- cell stages) were considered as degenerated and remaining with normal morphology considered as transferable.

The retarded embryos could have resulted from either late ovulations or an arrest in their developmental process may have potential to develop further if asuitable uterine environment can provided [4]. These values compared favorably with the data reportedearlier by [28] inferred that high estrogen output from large un-ovulated follicles increased the rate of sperm transport and culminating in low rates of embryo recovery. While on Day8 expects to find early, late and expanded blastocyst, this was the situation in this study. So it has been suggested that somatic cells may facilitate growth of embryos by reducing O2 tension and /or increasing concentrations of CO2 in the vicinity of embryos [29]. Studies using specifically labeled glucose indicate that the pentose phosphate pathway is the principle pathway for for the released of energy by the glucose in bovine embryos [19], [20], [21]. [21] suggested that glycosis is blocked in the bovine blastocyst due to lack or inhibition of Pyruvate kinase.The results of the present study will not support this suggestion,since loaf lactate were produced from the morula to the hatched blastocyst stage. Although bovine embryos utilized very little glucose until the 12- cell stage, there was no evidence for inhibitory effects of glucose on early embryonic development in the present study.

Overall analysis of embryos quality

An overall analysis of embryos quality, without seasonal consideration, in both groups morulae and blastocysts were recovered only from cows, on day 8 post estrus flushing. The located, embryos were classified according to International Embryo Transfer Society (IETS) guidelines [25].Embryos were graded as excellent, good, fair and degenerated and only excellent and good embryos selected for studies on energy requirement.

CO_2 production and substrate carbon uptake by embryos

The data obtain for CO_2 production and substrate carbon uptake from all three energy substrates by embryos derived invivo and developed further invitro was presented in table3). The invitrorequirement of energy substrates of 8- cell embryos derived invivo subjected to comparative studies of the same stage embryos produced invitro, it was inferred that, while the pattern of utilization of substrates was similar between embryos derived in vitro or in vivo, however, early embryo upto the 8-cell stage oxidized higher amounts of lactate and had a lower ratio of Pyruvate to lactate oxidation than their in vivo counterparts. Since lactate can only produce energy after being converted to Pyruvate and require NAd for this conversion, the above finding would suggest differences in ATP/ADP ratio and oxidation reduction status of the embryos grown in vitro or in vivo. It is possible that lactate plays a supporting role by providing a substrate pool from which Pyruvate could be continuously generated. However, it has been reported that lactate alone can support development of early bovine embryos [30], [31] was similar between embryos, oxidation and CO2 production of lactate in vitro was significantly higher compared with in vivo in the 8- cell embryos(c versus d=P =0.005).In general the two concentrations of glucose did not differ in their metabolic fate, although the incorporation tended to be higher in the presence of o.56 mmol/L.No lactate production was detected apart from the higher oxidation of lactate by the in vivo derived embryos of both stages, the metabolic capacity of embryos obtained from the two sources was similar(table III).

Table III: Metabolism of energy substrate by 8-cell embryos that were derivedinvitroorin vivo

Energy substrate	**In Vitro**	**In Vivo**
(a) Production of CO_2 (p moles)		
Pyruvate	2.54±0.26	2.29±0.46
Lactate Glucose	2.32±0.49[c]	0.59±0.80[d]
0.28mmol/L	0.31 ±0.10	0.23±0.05
0.56mmol/L	0.38±0.80	NP
(b) Incorporation(pg atoms)		
Pyruvate	1.29±0.04	1.33±0.93
Lactate Glucose	1.55±0.38	0.79±0.13
0.28mmol/L	3.60±0.70[a]	4.53±0.24[b]
0.56mmol/L	4.78±0.59[b]	NP

Values are means±SEM (per embryo per hour) for five replicates;

Means with different superscripts(a,b,c,d) in the each row are different(P < 0.005:

NP: not performed.

The invivo collected morulae and early blastocysts produced lower rates of CO_2 ($P < 0.05$) than their counterparts derived in vitro. Similarly, the mid blastocysts had a higher production of CO_2 in compared to the freshly collected mid blastocysts (table IV).

Table IV Comparative metabolism of glucose by embryos producedin vitro (IVM/IVF), obtained by flushing the uterus and used immediately (fresh) or obtained from the uterus and culturedin vitrofor 1–3 days (fresh+IVC)

Biochemical Parameter/ source of embryo	**Morula**	**Stages Early blastocyst**	**development Mid blastocyst**	**blastocyst**	**Hatched blastocyst**
			(a) Catabolic Utilization (I) CO_2 (p moles/hr)		
IVM/	7.20±1.50[a]	8.71±1.90[a]	8.00±0.64	9.90±1.50	10.85±1.40
Fresh	3.10±0.55[b]	4.65±0.21[b]	7.40 ±0.50[a]	NP	NP

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Fresh+IVC	NP	NP	10.33 ±0.38[b]	9.34±0.32	10.45±0.75
			(II) Lactate Production (p moles/hr)		
IVM/IVF	24.88±5.57[a]	26.30±6.30[a]	45.94±7.60[b]	69.24±4.50[a]	69.14±8.63
Fresh	10.90±1.40[b]	10.32±1.43[b]	22.60±5.97[a]	NP	NP
Fresh+IVC	NP	NP	40.54±1.55[b]	39.62±8.65[b]	57.13±20.94
			(b) Incorporation (p mole/hr)		
IVM/IVF	17.73±4.13	18.00±5.07	17.92±2.04[a]	23.71±4.52	21.88±2.01
Fresh	13.69±1.76	16.50±0.73	20.00±10.58[a]	NP	NP
Fresh+IVC	NP	NP	46.43±10.67[b]	29.96±3.70	33.21±9.96

Values are means ±SEM (per embryo per h); NP: not performed a,b differ significantly within biochemical parameter in each row or column of a stage of development ($p<0.05$)

IV. Discussion

In the present study, irrespective to season1s, observed that the majority of cows exhibited estrus 8 hr following removal of ear implant and in the summer season 6 cows exhibited estrus 18 hr following estrus.

In the present study, a reasonably good superovulatory response was obtained during winter season, but the slightly lower response in the summer season is similar to the results reported by[32],they reported significantly higher ovulation rates with the decreasing dose schedule of FSH during the winter season. The present study compare with the statements reported by[33].

Type of super stimulatory agents (FSH-E), and the effects induced by Gn-RH affect the ovulatory response. They observed that, breed can affect the ovulation rate, attempts to minimized the variation in ovulation time have been made either using Gn-RH or hCG by various researchers and as compared to results reported in previous study [33].

In conclusion, season, breed, time of estrus expression in cross bred cows did not alter number of transferable embryos. The most important conclusion in present study, when were given exogenous Gn-RH was the uniformity in the percentage of ovulating follicles which apparently sufficient LH would be available to cause ovulation. This finding leaves the unanswered question of; whether similar rates of ovulation would have been obtain if Gn-RH was not administered. However, In this study, i noticed season and time of onset of estrus did not affect number of transferable embryos in cross bred cows ., but results of this study indicate cross bred cows exhibit a lower ovulatory response compared to results reported by [34].[34]reported a Progress in understanding ovarian follicular dynamics in cattle. So [35],[36],[37] introduced an alternative approaches to setting up donor cows for super stimulation and shown a new approaches to superovulation in the cow and Simplification of superovulation protocols in cattle was advocated.

The viability of the embryos subjected to the measurements of energy requirement was examine by returning 10 embryos at the 2-cell stage to co-culture with cumulus cells after measuring their CO_2 production.2 of these (20%) progressed to the morula/early blastocyst stage. This proportion is low, but within the range observed for in vitro produced embryos in the present study. The composition of the medium used for the measurements of energy consumption, in no way is resembled the composition of the genital tract fluids. Other control mechanisms operating in vivo were lacking during the in vitro measurements.

The concentration of glucose (0.28mmol/L) used in the bulk of the present experiments to measure energy consumption by embryos. The overall pattern of glucose requirement from the 1-cell to late preimplantation stages to that reported for cattle embryos derived in vitro or in vivo[19],[20]. [20] reported a 30 times increase in the total glucose consumption of glucose by bovine embryos during in vitro development from the 2-cell to the expended stage. The first marked increase in the utilization of glucose occur between the 8-cell and 16-cell stages[20] .Since the present study also included 12-cell stage embryos and oxidation of glucose did not differ between the 8-cell and 12-cell stages ,therefore, the first significant increase in oxidation of glucose of bovine embryos have occure between the 12 and16 cell stages. However [19] reported that first marked increase in the oxidation of glucose could be detected between the 16-cell and morula stages in bovine embryos collected from super ovulated animals.

Although the two concentrations of glucose did not differ in their oxidation fate, but the incorporation tended to be higher in the presence of o.56mmol/L. However production of lactate and uptake of glucose carbon were higher in the presence of 0.56mmol/L glucose than o.28mmol/L.No lactate production was detected apart from the higher oxidation of lactate by the invivo derived embryos of both stages (table4).

The invivo collected morulae and early blastocyst produced lower rates of CO_2 than their counterparts derived in vitro. Similarly, the mid blastocyst for 24hrhad a higher production of CO_2 in compared to the freshly collected mid blastocyst .In general, lactate production by the freshly obtained embryos was about one half of the invitro generated embryos. However, long pre-exposure to culture conditions narrowed this gap and the differences between the two sources at the hatched blastocyst stage (72 hr of IVC) were not significant. Incorporation of glucose carbon was independent of the source of embryos but there was a tendency for a lower uptake by IVM/IVF embryos from the mid blastocyst to the hatched blastocyst. (table5).

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A proper lactate to Pyruvate ratio is essential for balancing the oxidation/reduction potential in embryos. It is possible that lactate plays a supporting role by providing a substrate pool from which Pyruvate could be continuously generated. However, it has been reported that lactate alone can support development of early bovine embryos [24,25]. Both lactate and Pyruvate can affected oxidation of each other, it is possible that the presence of somatic cells and glucose during in vitro culture of bovine embryos ratio, because the cumulus cells are capable of producing Pyruvate.This suggestion is supported by a study [35], in which Pyruvate was required for meiotic maturation in denuded but not in cumulus enclosed bovine oocytes. Based on the present results, a low Pyruvate to lactate ratio should be beneficial during embryo culture in chemically defined media. Some studies have used a low Pyruvate to lactate ratio for culture of cattle embryos [38] ,with development rates equal to those obtained with complex culture conditions and a high lactate to Pyruvate ratio.

In conclusion, based on the present results, the production of CO2 from glucose by embryos produced in vitro or obtained in vivo was similar. However, only one embryo reached to the maximum rate of development earlier in vitro embryos than in fresh embryos. So, it inferred that, low pyruvate to lactate ratio should be beneficial during cultured in chemically defined media. One of thereasons for the high lactate production could be stress response to a sub optimal cultured environment. The culture environment which was provided optimum development were due to variability in the PH and oxygen tension, which in this experiment could not maintained constant throughout the experiment. Both lactate and pyruvate affected metabolism of each other, as has been reported earlier for mouse embryos. In the presence of 25mmol/L lactate, utilization of pyruvate (0.5mmol/L) was drastically reduced, arising seriously doubts about the needs of high amounts of lactate in culture medium.

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