BIOLOGY OF REPRODUCTION 46, 470–474 (1992)

Human Leukemia Inhibitory Factor Improves the Viability of Cultured Ovine Embryos[1]

R. C. FRY,[2,3] P. A. BATT,[4] R. J. FAIRCLOUGH,[3] and R. A. PARR[3]

Victorian Institute of Animal Science,[3] Werribee, Victoria 3030, Australia
Centre for Early Human Development,[4] Monash Medical Centre, Clayton, Victoria 3168, Australia

ABSTRACT

Embryos were collected from ewes on Day 6 after estrus (Day 0 = estrus), placed in M2 culture medium, and assigned to 1 of 4 treatment groups. Some embryos were transferred to recipient ewes on Day 6 of their estrous cycle either in pairs (group 1) or singularly (group 2) within 3 h of collection. The remaining embryos were individually cultured for 48 h in an atmosphere of 5% CO_2 in humidified air in either synthetic oviduct fluid (SOF) medium (group 3) or SOF containing 1 000 U/ml of recombinant human leukemia inhibitory factor (hLIF) (SOF + hLIF: group 4). These embryos were then transferred to recipient ewes on Day 8 of their estrous cycle. The addition of hLIF to culture medium significantly improved the development of the embryos compared with control embryos prior to transfer (blastocysts hatching from the zona pellucida: group 3 = 16% vs. group 4 = 64%, $p < 0.05$; those degenerative: group 3 = 27% vs. group 4 = 9%, $p < 0.05$) and the subsequent pregnancy rates of the recipient ewes, receiving a single embryo, at Day 70 of pregnancy (group 3 = 16% vs. group 4 = 50%, $p < 0.05$). The pregnancy rate of ewes given embryos cultured for 48 h in SOF + hLIF prior to transfer (50%; group 4) was similar to the group 2 ewes receiving a single embryo soon after collection (52%), but the pregnancy rate for both groups was significantly lower than that for the group 1 ewes receiving two embryos soon after collection (89%: 53% twins, 36% singles; $p < 0.05$).

Downloaded from https://academic.oup.com/biolreprod/article/46/3/470/2763230 by guest on 16 August 2022

INTRODUCTION

Embryo transfer in the livestock industries is becoming a more widely adopted practice as new technologies are developed. One major constraint with embryo transfer is the need to hold embryos in culture medium for either relatively short periods of time—perhaps only a few hours prior to transfer—or for longer periods of some days after micromanipulation. Both practices substantially reduce the development of the embryo and its chances of survival [1].

The short-term collection and maintenance of embryos (usually morulae or blastocysts) in simple medium such as PBS appears adequate provided that the concentration of phosphate and potassium ions is correct [1]. Often when the embryos are held for some hours, more complex media containing the energy substrates pyruvate, lactate, and/or glucose are used. Other factors such as temperature, pH, and light are important [1].

Long-term culture usually applies to the culture of ova or early-stage embryos to the blastocyst stage of development following in vitro fertilization (IVF), nuclear transplantation, splitting or other manipulations, and assessment of embryonic viability prior to transfer. Success rates for the long-term culture of sheep, cattle, and goat embryos are often in the range of 30% [2–6]; this limitation severely compromises the application of new technologies. In sheep, goats, and cattle, few embryos develop in either simple or complex media through the 8–16-cell stage [1, 7], which is when the embryonic genome is switched on [8]. Adjustment of the atmospheric O_2 tension appears to allow embryos to pass through this block [9–11]. Furthermore, the culture of preimplantation embryos for periods of some days has a general debilitory effect on development, as shown by lower cell numbers [6], morphological development [12], and decreased pregnancy rates [3, 13], compared to embryos in vivo. Even the use of media formulated specifically for the natural environment of the embryo such as Menezo's medium [14] or synthetic oviduct fluid [2] has failed to completely overcome this problem. These data suggest that certain factors may be missing from the media, and these factors may vary with the changing stages of embryonic development [1, 15]. Coculture of embryos with oviduct epithelial cells [6, 13, 16] or trophoblastic vesicles [5] has been used to increase the developmental rate of embryos, but the active constituent(s) has yet to be defined.

Leukaemia inhibitory factor (LIF) is a glycoprotein, first isolated in mice, that induces differentiation and inhibits the proliferation of the M1 myeloid leukemia cell line [17, 18]. In contrast to this action, LIF can stabilize murine embryonic stem cells in culture, preventing them from undergoing spontaneous differentiation [19, 20]. LIF is produced by mouse endometrial glands (C. Stewart, pers. comm.) and receptors have been found on the 4-day-old mouse embryo (L. Williams, pers. comm.). These findings suggest that LIF may have a physiological role in the development of the embryo. In mice, the addition of murine LIF to culture media enhances the hatching process and increases the number of embryos undergoing the developmental changes in vitro necessary for implantation [21]. Our aim was to determine if the addition of human LIF to synthetic oviduct fluid (SOF) culture medium would improve the development and viability of ovine embryos collected on Day 6 and cultured for 48 h prior to transfer to recipient ewes.

Accepted November 1, 1991.
Received September 3, 1991.
[1]Supported by the AMRAD Corporation, Kew, Victoria, Australia.
[2]Correspondence: Dr. Richard C. Fry, Victorian Institute of Animal Science, Werribee, Victoria, 3030 Australia. FAX: (61)-3-7420200

MATERIALS AND METHODS

Embryo Collection

Forty mature Merino ewes were treated for 14 days with a controlled internal drug-releasing device (CIDR) containing 0.3 g progesterone (Riverina Artificial Breeders, NSW, Australia) and were superovulated by the injection (i.m.) of 5 mg of the pituitary follicle-stimulating hormone FSH-P (Intervet Australia, Sydney, NSW, Australia) and 200 IU of eCG (Pregnecol, Heriot Agvet, Melbourne, Victoria, Australia) 48 h before CIDR withdrawal, followed by decreasing doses of FSH-P (4, 3, 3, 2, and 1 mg) every 12 h afterwards [22]. Animals also received a 50-μg injection (i.m.) of GnRH (Auspep, Melbourne, Victoria, Australia) 24 h after CIDR withdrawal, which coincided with the time that estrus was detected in most ewes by vasectomized rams fitted with marking crayons. Ewes were inseminated via a laparoscope with approximately 100×10^6 fresh motile sperm into each uterine horn 8–12 h after estrus. Semen was collected from 4 fertile rams, diluted 1:1 in PBS (Flow Laboratories, North Ryde, NSW, Australia) and kept at ambient temperature prior to insemination. Six days after estrus, the ewes were anesthetized with an injection (i.v.) of the barbiturate sodium thiopentone (Intraval, May & Baker, Footscray, Victoria, Australia) and maintained under general anesthesia by a mixture of halothane (May & Baker) and oxygen. The uterus was exteriorized by a mid-ventral laparotomy and each horn was cannulated with an 8-gauge Foley catheter (Promedica, Moorabin, Victoria, Australia) at approximately 1 cm below the bifurcation of the uterine horns. A 20-gauge needle attached to a 20-ml syringe filled with flushing medium was passed into the lumen of the uterus near the utero-tubal junction. Each horn of the uterus was flushed and the medium plus embryos was collected into glass vessels via a Foley catheter. Embryos were pooled in M2 holding medium containing 4 mg/ml BSA (Pentex Crystalline, Fraction V, Miles Diagnostics, Kankakee, IL) and graded according to quality and stage of development before allocation to treatment groups.

Culture of Embryos

Embryos (166 assessed as good quality and 17 as poor quality) were randomly allocated to one of four treatment groups. Embryos in treatments 1 and 2 (80 good and 5 poor quality embryos at the morula or early blastocyst stage) were cultured in lots of 10 for 1–2 h in 5 ml of M2 holding medium prior to being transferred either as pairs (group 1; n = 38) or singly (group 2; n = 47) into recipient ewes at Day 6 of the estrous cycle. Group 3 embryos (44 good quality and 6 poor quality embryos at the morula or early blastocyst stage) were cultured individually in SOF medium supplemented with 32 mg/ml BSA for 48 h prior to transfer. Three separate 100-μl droplets of SOF culture medium were placed in a 35×10-mm plastic Petri dish and covered with 2.5 ml paraffin oil (Labchem, Ajax Chemicals, Auburn, NSW, Australia). One embryo was then placed in each droplet and the Petri dishes were placed in a water-jacketed incubator and were maintained at 39°C in an atmosphere containing 5% CO_2 in humidified air. Group 4 embryos (44 good quality and 6 poor quality morulae or early blastocysts) were cultured for 48 h in SOF culture medium as for group 3, except that human recombinant LIF was added to the medium at a rate of 1 000 U/ml.

The number of good quality or poor quality (degenerating) embryos—those achieving morula, blastocyst or hatching blastocyst stage of development—was recorded at the time of embryo collection, every 12 h afterwards, and immediately prior to transfer to recipient ewes at Day 8 after estrus.

Culture Media

The flushing medium used was Dulbecco's PBS containing 10% fetal calf serum, Penicillin G, potassium salt (0.060 g/L), and streptomycin sulphate (0.050 g/L).

M2 holding medium [23] was a modified Krebs-Ringer solution with some of the bicarbonate substituted with HEPES buffer; the SOF culture medium was made according to Tervit et al. [2].

All media were sterilized by filtration through a 0.2-μm filter (Millipore Pty. Ltd., Richmond, Victoria, Australia).

Embryo Transfer

The estrous cycles of 200 3-yr-old maiden Merino ewes were synchronized with a 14-day CIDR treatment. An injection of 400 IU eCG was given at the time of CIDR withdrawal. Vasectomized rams, fitted with harnesses and crayons, were placed with the ewes to detect estrus. Ewes observed in estrus were randomly allocated to one of four recipient groups. Recipient ewes were treated with local anesthetic (lignocaine, Lyppard Chemicals, Brighton, Victoria, Australia) and laparoscopic procedures were used to determine the number of corpora lutea on each ovary. The uterus was located and the tip of the uterine horn ipsilateral to an ovulating ovary was exteriorized through a 2-cm mid-ventral incision. The uterus was punctured approximately 4 cm from the utero-tubal junction and the embryo(s), bathed in M2 holding medium, was deposited via a Tomcat catheter (Size 3.5 FR, Lyppard Chemicals) attached to a 1-ml syringe. The uterine horn was returned into the abdominal cavity and the incision was sutured.

All recipient ewes were released to graze with harnessed vasectomized rams for 21 days to provide an estimate of embryonic loss prior to or at implantation [24]. Ewes were scanned using ultrasonics on Day 70 of pregnancy to determine the number of healthy fetuses.

Analysis

Nonparametric differences between groups were determined by Chi-square analysis.

Downloaded from https://academic.oup.com/biolreprod/article/46/3/470/2763230 by guest on 16 August 2022

RESULTS

Embryo Culture

The addition of 1 000 U/ml of human recombinant LIF to SOF culture medium significantly improved the development (more blastocysts hatching and less degenerating) of good quality morula and blastocyst embryos cultured for 48 h in vitro (Table 1).

Both treatment groups had 6 embryos initially classified as poor quality at the time of embryo recovery. Over the treatment period their health did not improve so they were discarded and not transferred to recipient ewes.

Pregnancy Rates

The pregnancy rate (as determined by real-time ultrasonic scanning on Day 70 of pregnancy) of ewes receiving an embryo cultured for 48 h in SOF alone (group 3) was significantly lower than that of ewes receiving a single embryo within 3 h of collection (group 2: Table 2). In contrast, the addition of hLIF to the medium maintained the viability of the embryos during 48 h of culture so that the pregnancy rate of ewes receiving these embryos (group 4) was not different from those ewes receiving an embryo soon after collection (group 2). The pregnancy rates of both of these groups (2 and 4) were lower than that of recipient ewes that had 2 embryos transferred soon after collection (group 1). Although only 29% of transferred embryos were lost in group 1 compared to about 50% in both groups 2 and 4, these differences were not significant ($p > 0.05$).

The 21-day nonreturn to service rate (an indicator of embryo survival to Day 12 [24]) reflected the Day 70 pregnancy data, indicating that the major effect of hLIF occurred prior to implantation (Table 2).

TABLE 2. Percentage of recipient ewes not returning to service by Day 21 and percentage of ewes pregnant on Day 70 after estrus.

Treatment	n	Day 21 (%)	Day 70 (%)
Group 1: 2 embryos <3 h in M2	19	100[a]	89[a]
Group 2: 1 embryo <3 h in M2	42	68[b]	52[b]
Group 3: 1 embryo 48 h in SOF	44	41[c]	16[c]
Group 4: 1 embryo 48 h in SOF + LIF	42	64[b]	50[b]

[abc]Means within columns differ significantly ($p < 0.05$).

DISCUSSION

The addition of hLIF to culture medium increased by 4-fold the number of sheep blastocysts that hatched from the zona pellucida after culture for 48 h. Fewer embryos became degenerative when cultured in medium containing hLIF. These results were reflected in pregnancy rates when the embryos were transferred to recipient ewes. Overall, our findings indicate that hLIF has potential as either an embryotrophic or embryo-protective agent.

Media for culturing preimplantation embryos have been developed to optimize embryonic growth and to maximize the number of embryos that survive after transfer. Significant advances have been made over the last 20 years through formulations based on oviduct fluid [2, 14], correcting the oxygen tension [9, 25], and by providing the embryo's specific metabolic requirements [26, 27]. Although embryos from most species of mammals can now be cultured from the one-cell stage to blastocysts, the viability of these embryos is severely compromised [1, 29] and the development of Day 5 ovine embryos cultured for 2 days is retarded [23]. These data indicate that one or more factors are missing from the artificial media that are present during in vivo embryonic development.

When hLIF was added to culture medium containing 8-cell murine embryos, there was a marked increase in the number of embryos completing the developmental changes associated with implantation [21]. Our results demonstrate that the addition of human recombinant LIF to culture media will increase up to 4-fold the number of ovine blastocysts that hatch from the zona pellucida and decrease the number of embryos degenerating during their 48-h culture in SOF in an atmosphere of 5% CO_2 in humidified air. In fact, 64% of the embryos cultured in SOF + hLIF had hatched by Day 8 after estrus, which is similar to the rate found in vivo [29, 30]. The pregnancy rates of the recipient ewes receiving an embryo cultured in medium containing hLIF for 48 h was equal to, but not higher than, that of ewes receiving an embryo immediately after collection, indicating that hLIF may have a role in maintaining the viability of

TABLE 1. Number and percentage of healthy embryos hatching or degenerating during 48-h culture in SOF (n = 42) or SOF + 1 000 units/ml hLIF (n = 44).

Time in culture	Hatched Embryos				Degenerating Embryos			
	SOF		SOF + hLIF		SOF		SOF + hLIF	
	Number	%	Number	%	Number	%	Number	%
0 h	0	0	0	0	0	0	0	0
12 h	0	0	3	7	2	5	0	0
24 h	1	2	10*	23	2	5	1	2
36 h	6	14	19*	43	5	11	1	2
48 h	7	16	28*	64	13*	27	4	9

*Denotes significant differences between SOF and SOF + hLIF treatment groups $p < 0.05$.

Downloaded from https://academic.oup.com/biolreprod/article/46/3/470/2763230 by guest on 16 August 2022

embryos under adverse conditions rather than improving their development. Furthermore, no embryos deemed degenerative prior to culture were rescued in the medium containing hLIF. It is interesting to note that mLIF does not appear to have an effect on cultured ovine embryos (R.F. Seamark, pers. comm.; R.C. Fry and R.A. Parr, unpublished results). This may be due to the higher degree of homology between natural ovine LIF and hLIF than between ovine LIF and murine LIF (P.A. Willson and N.H. Gough, unpublished results).

This action of hLIF on the development of ovine embryos is coincidental to the period (Day 4) that murine LIF is expressed strongly by the endometrial glands of the pregnant and pseudopregnant mouse (C. Stewart, pers. comm.) and receptors found on the embryo (L. Williams, pers. comm.). LIF therefore appears to be produced between the time that the embryo enters the uterus from the oviduct and implantation, so LIF may play a role in supporting the survival of the embryo during this critical time.

Currently, in most embryo transfer programs in both humans and livestock, more than one embryo is transferred to each recipient to ensure a viable pregnancy. One notable exception is the cow where usually only one embryo is transferred to avoid the possibility of freemartins. There is a possibility that the inclusion of LIF in transfer medium may improve the ability of the embryo to implant and make the transfer of a single embryo to each recipient both desirable and practical. Other substances that could have a role in regulating embryo survival include ovine trophoblast protein [31, 32], macrophage colony-stimulating factors [33], platelet-activating factor [34], and plasminogen [35]. Plasminogen has displayed similar embryotrophic effects to LIF, although it is produced by the embryo.

LIF has very diverse effects upon cells, which are largely determined by the target cell. For example, while LIF inhibits differentiation of murine embryonic stem cells [19], it induces the differentiation of Ml myeloid leukemia cells [18]. Medium containing LIF could be used for early manipulative procedures on the oocyte/embryo such as in vitro fertilization, embryo splitting, and nuclear transplantation, where survival rates of embryos are low. LIF may also have important applications in the growth of totipotent stem cell lines for cloning [36], or perhaps for inclusion into the medium used for the transport of cooled or frozen embryos/semen.

ACKNOWLEDGMENTS

The authors wish to thank Dr A.W.N. Cameron for helpful discussion and Ms. T. Purdon and Messrs. D. Kerton, T. Squires, and P. Weston for expert technical assistance. The AMRAD Corporation, Kew, Victoria, Australia, donated the hLIF.

REFERENCES

1. Bavister BD. Role of oviductal secretions in embryonic growth in vivo and in vitro. Theriogenology 1988; 29:143–154.
2. Tervit HR, Whittington DG, Rowson LEA. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 1972; 30:493–496.
3. Tervit HR, Rowson LEA. Birth of lambs after culture of sheep ova in vitro for up to 6 days. J Reprod Fertil 1974; 38:177–179.
4. Wright RW, Anderson GB, Cupps PT, Drost M. Successful culture in vitro of bovine embryos to the blastocyst stage. Biol Reprod 1976; 14:157–162.
5. Heyman Y, Menezo Y, Chesne P, Camous S, Garnier V. In vitro cleavage of bovine and ovine early embryos: improved development using coculture with trophoblastic vesicles. Theriogenology 1987; 27:59–68.
6. Sakkas D, Batt PA, Cameron AWN. Development of preimplantation goat (*Capra hircus*) embryos in vivo and in vitro. J Reprod Fertil 1989; 87:359–365.
7. Wright RW, Bondioli KR. Aspects of in vitro fertilization and embryo culture in domestic animals. J Anim Sci 1981; 53:702–729.
8. Crosby IM, Gandolfi F, Moor RM. Control of protein synthesis during early cleavage of sheep embryos. J Reprod Fertil 1988; 82:769–775.
9. Thompson JGE, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen concentration on in vitro development of preimplantation sheep and cattle embryos. J Reprod Fertil 1990; 89:573–578.
10. McLaughlin KJ, McLean DM, Stevens G, Ashman RA, Lewis PA, Bartsch BD, Seamark RF. Viability of one-cell bovine embryos cultured in synthetic oviduct fluid medium. Theriogenology 1990; 33:1191–1199.
11. Batt PA, Gardner DK, Cameron AWN. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. Reprod Fertil Devel 1991; 3:601–607.
12. Quinn P, Warnes GM, Walker SK, Seamark RF. Culture of preimplantation sheep and goat embryos. In: Lindsay DR, Pearce DT (eds.), Reproduction in Sheep. Canberra: Australian Academy of Science; 1984: 289–290.
13. Gandolfi F, Moor RM. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J Reprod Fertil 1987; 81:23–28.
14. Menezo Y. Milieu synthetique pour la survie et la maturation des gametes et pour la culture de l'oeuf feconde. CR Acad Sci D Paris 1976; 282:1967–1970.
15. Betteridge KJ. The anatomy and physiology of pre-attachment bovine embryos. Theriogenology 1988; 29:155–187.
16. Eyestone WH, Leibfreid-Rutledge ML, Northey DL, Gilligan BG, First NL. Culture of one- and two-cell bovine embryos to the blastocyst stage in the ovine oviduct. Theriogenology 1987; 28:1–7.
17. Gearing DP, Gough NM, King JA, Hilton DJ, Nicola NA, Simpson RJ, Nice EC, Kelso A, Metcalf D. Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). EMBO J 1987; 6:3995–4002.
18. Gough NH, Gearing DP, King JA, Willson TA, Hilton DJ, Nicola NA, Metcalf D. Molecular cloning and expression of the human homologue of the murine gene encoding for myeloid leukaemia-inhibitory factor. Proc Natl Acad Sci 1988; 85:2623–2627.
19. Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wagner E.F, Metcalf D, Nicola NA, Gough NM. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 1988; 336:684–687.
20. Gearing DP, Nicola NA, Metcalf D, Foote S, Willson TA, Gough NM, Williams RL. Production of leukaemia inhibitory factor in *Escherichia coli* by a novel procedure and its use in maintaining embryonic stem cells in culture. Bio/Technology 1989; 7:1157–1161.
21. Robertson SA, Lavranos TC, Seamark RF. In vitro models of the maternal fetal interface, In: Wegmann TG, Nisbett-Brown E, Gill TJ (eds.), The Molecular and Cellular Immunology of the Maternal-Fetal Interface. 1990; 111:191–206.
22. Ryan JP, Bilton RJ, Hunton JR, Maxwell WCM. Superovulation of ewes with a combination of PMSG and FSH-P. In: Lindsay DR, Pearce DT (eds.), Reproduction in Sheep. Canberra: Australian Academy of Science; 1984: 338–341.
23. Quinn P, Barros C, Whittington DG. Preservation of hamster oocytes to assay the fertilising capacity of human spermatozoa. J Reprod Fertil 1982; 66:161–168.
24. Edey TN. Early embryonic death and subsequent cycle length in the ewe. J Reprod Fertil 1967; 13:437–443.
25. Weitlauf HM. Biology of implantation. In: Knobil E, Neill J (eds.), The Physiology of Reproduction. New York: Raven Press; 1988: 231–262.
26. Reiger D, Guay P. Measurement of the metabolism of energy substrates in individual bovine embryos. J Reprod Fertil 1988; 83:585–591.
27. Gardiner DK, Sakkas D. The effects of ions and metabolites on the development, metabolism and viability of mouse embryos in culture. In: Proc Aust Soc Reprod Biol 1990; 111 (abstr.).
28. Walker SK, Lampe RJ, Seamark RF. Culture of sheep zygotes in synthetic oviduct medium with different concentrations of sodium bicarbonate and HEPES. Theriogenology 1989; 32:797–804.
29. Rowson LEA, Moor RM. Development of the sheep conceptus during the first fourteen days. J Anat 1966; 100:777–785.

Downloaded from https://academic.oup.com/biolreprod/article/46/3/470/2763230 by guest on 16 August 2022

30. Bindon BM. Systematic study of preimplantation stages of pregnancy in the sheep. Aust J Biol Sci 1971; 24:131–147.
31. Martal J, Lacroix M-C, Loudes C, Sannier M, Wintenberger-Torres. Trophoblastin, an antiluteolytic protein present in early pregnancy in sheep. J Reprod Fertil 1979; 56:63–73.
32. Godkin JD, Bazer FW, Moffatt RJ, Sessions F, Roberts RM. Purification and properties of a major low molecular weight protein released by the trophoblast of sheep blastocysts at Day 13–21. J Reprod Fertil 1982; 65: 141–150.
33. Pollard JW, Bartocci A, Arceci R, Orlofsky A, Ladner MB, Stanley ER. Apparent role of the macrophage growth factor, CSF-1 in placental development. Nature 1987; 330:484.
34. O'Neil C. Embryo derived platelet activating factor: a preimplantation embryo mediator of maternal recognition of pregnancy. Dom Anim Endocrinol 1987; 4:69–85.
35. Menino AR, Dyk AR, Gardiner CS, Grobner MA, Kaaekuahiwi MA, Williams JS. The effect of plasminogen in vitro ovine embryo development. Biol Reprod 1989; 41:899–905.
36. Li R, Trounson A. Establishment of sheep embryonic stem cells: observation of cell lines derived from frozen-thawed blastocysts. In: Aust Soc Reprod Biol 1990; 51 (abstr.).

Downloaded from https://academic.oup.com/biolreprod/article/46/3/470/2763230 by guest on 16 August 2022