Ethicolegal Aspects of Reproductive Science:

Adapting the 14-day rule for embryo research to encompass evolving technologies

Abbreviations:
HFE: Human Fertilisation and Embryology
HFEA: Human Fertilisation and Embryology Authority
IVF: *in vitro* fertilisation
hESC: human embryonic stem cells
SC: stem cell
IVC: *in vitro* culture medium
HCS: human cord serum
KSR: KnockOut Serum Replacement
PGC: primordial germ cells
EMT: epithelial-mesenchymal transitions
TSC: trophoblast stem cells
XEN: extraembryonic endoderm or hypoblast stem cells in mice
BMP4: bone morphogenetic protein 4
PASE: post-implantation amniotic sac embryoid
HDAC: histone deacetylase
ROCK: Rho-associated protein kinase
ISSCR: International Society for Stem Cell Research

The 14-day rule, established in the UK's Warnock Report of 1984, and then in the HFE Act 1990, prevents the *in vitro* culture of human embryos beyond 14 days after embryo creation, acting as an ethical safety barrier. It is regulated by the HFEA, and was developed in response to the introduction of IVF in 1978, when the ability to develop embryos *in vitro* for research and assisted reproductive therapy was demonstrated. The 14-day rule is now followed by law in at least 12 countries, and by five other countries under national scientific guidelines [1]. Therefore, it is one of the most internationally accepted rules in reproductive medicine [2].

The placement of the boundary originated because the primitive streak appears on day 15, as an objective visible streak of cells, marking the onset of gastrulation (a period of cell internalisation forming the 3 germ layers - endoderm, ectoderm and mesoderm- as well as the body form and axes), which signifies that an 'individual' embryo is now evident and that it can no longer split to form twins.

The original HFE Act 1990 prohibited 'using an embryo after the appearance of the primitive streak', which is deemed to not be after 14 days since gamete mixing [3]. This was changed in 2008 to: no culture beyond 14 days since 'creating the embryo began', and applies to all 'live human embryos regardless of the

manner of their creation, and to all live human gametes', thus updating the Act to account for new technologies, e.g. certain cloning techniques, and to permit the creation of 'admixed' embryos (combined human and animal genetic material) for research purposes [4]. This shows that successful adaptation to follow scientific advances is possible.

In 1990, 14 days seemed plenty of time, since embryo culture *in vitro* was limited. However, now, due to scientific advances, the 14-day rule seems too restrictive. I will argue that the limit needs to be changed, based on ongoing embryological research, and will propose how a new limit should be positioned.

Recent discoveries researching human embryos

Whilst many discoveries have been made within the 14-day culture limitation, two main advances have been made recently, showing that embryos are more self-sufficient than previously believed. In 2016, two groups (led by Prof. Zernicka-Goetz, and Dr. Brivanlou) reported successful *in vitro* human embryo growth to 13 days (gastrulation), via new protocols, past the original hurdle of 7 days (implantation), showing they had created a maternal environment *in vitro*. This means we could potentially analyse gastrulation, highlighting how a free-floating blastocyst becomes an implanted gastrula. This achievement came after a 2014 breakthrough, which discovered that hESCs can self-organise into structures resembling post-implantation embryos ('embryoids') [5].

Zernicka-Goetz's laboratory managed initially to culture mouse embryos *in vitro* past implantation, highlighting that the epiblast's self-organisation into rosettes with a pro-amniotic cavity was due to extraembryonic (integrin/laminin) signalling, not apoptosis [6]. Moulding this protocol to human embryos, they found information from monkey embryo culture more useful than mouse [7].

Although mouse and human pre-implantation embryos morphologically develop similarly, they do so at different rates [8], and differ structurally after implantation [9], illustrating why, to establish clinical benefits, we will need to research human embryos/'embryoids'.

The human methodology was elucidated [10, 11], embryos being cultured on plastic microplates (for time-lapse confocal microscopy) with IVC1 initially, followed by IVC2 [10] (modified from IVC1 by substitution of HCS with KSR, a growth-promoting serum substitute), using 21% oxygen rather than the commonly used

IVF conditions of 5% oxygen. They achieved the major landmarks of successful post-implantation development (elucidated from Carnegie specimens) in 30% of cultured embryos [9]. This is an amazing achievement, with potential to increase this percentage with extension to the Act.

Scientific and clinical advances to be gained beyond 14 days

Extending the limit would allow *in vitro* studies elucidating events of the 'black box' (day 7-28, where the embryo is implanted and thus hard to study), studying gastrulation, the establishment of major organs, cell fate decisions, EMTs, the origin of PGCs [2], and morphologically how the embryonic disc folds ventrally. This period holds clear clinical benefits because days 14-28 are when embryological defects tend to occur. Organogenesis, when the embryo is most sensitive to teratogens, begins around 21 days. Additionally, before 4 weeks, the mother may be unaware of the pregnancy, making alcohol/drug-induced defects more likely [12], with almost 50% of fertilised eggs dying in this period, and 20-25% dying after this before seven weeks [12, 13]. Additionally, 70% of IVF conceptions fail [14]. Research on teratogens, miscarriages and defects could enhance pregnancy rates and IVF success, by finding markers of incompetent embryos and using CRISPR-Cas9 to elucidate gene function. As PGCs are specified around week 2.5, extending the Act could aid understanding of germline cancers and how PGCs and the early embryonic environment transfer epigenetic programming to future generations and affect adult health [12]. It would also enhance regenerative medicine via improved production of functional differentiated cells.

Embryoids

The importance of understanding gastrulation has led researchers to find alternatives to *in vitro* embryo culture, e.g. embryoids made from hESCs. Elucidated in mice first, murine ESC, TSC and XEN cell lines were used to create the most realistic 'ETX' embryoids [15]. These experiments began using ESC culture alone [16] but correct epiblast morphogenesis (rosette) failed, due to lack of interactions between cell types. Human embryoids proved harder. Like mice embryoids, they first cultured hESCs alone, which formed disorganised embryoid bodies [17]. But, for years, researchers found no human TSCs/XEN cells. Instead, they took inspiration from studies of non-human primate ESCs [18], seeding hESCs onto circular

micropatterns to support clustering and self-organisation of 2D structures [5]. With addition of BMP4, concentric rings formed, containing cells of the three germ layers, due to receptor exposure and production of BMP inhibitor, Noggin. By addition of activin-A/Nodal and WNT3A, a functional human 'organiser' formed, which patterns the head-tail axis [19], the existence of which was previously undetermined in humans, showing the benefits of embryoids. Although micropatterned embryoids lack an amnion/amniotic cavity, the PASE method of forming a human embryoid [20] induces these via an ECM and substrate gel bed. PASE embryoids are currently less reproducible than micropatterned embryoids, so these strategies need to be developed.

Bioprinting and other future developments

Additionally, there are new technologies on the horizon: derivation of human extra-embryonic cell lines is underway. Human TSCs have been derived recently [21] by supplying Wnt and EGF, with inhibition of TGFβ, HDAC and ROCK, allowing maintained culture of human villous cytotrophoblast cells. Additionally, Zernicka-Goetz's laboratory is attempting to derive hypoblast SC lines (like mouse XEN cells). Therefore, perhaps human ETX embryoids are on the horizon, replicating mouse protocols for human embryoids. Another key developing technique is 3D bioprinting, using coordinates to precisely position different cell types and deposit matrix, printing the required structure in consecutive layers. Although organ tissue has been bioprinted, functional organs have not, due to a need to improve printer resolution and speed [22]. Could this result in the printing of embryoids? hESCs (bioink) had seemed too delicate to print, but Scotland-based scientists [23] have developed a protocol for overcoming this. Another issue is the vasculature of the organ/embryoid, as diffusion is insufficient, and mixing bioink with angiogenic factors is difficult to regulate [22]. Therefore, co-printing with several bioinks could be used to integrate vasculature into cell cultures, perfusing the vasculature externally [24].

A proposal

Why does the law regulate *in vitro* and *in vivo* embryos so differently [25]? Research embryos must be terminated at 14 days to prevent individuation, whereas *in vivo* embryos can be terminated up to 24

weeks if continuance 'would involve risk, greater than if the pregnancy were terminated, of injury to the physical or mental health of the pregnant woman or any existing children' [26]. Objectively more people will benefit from extended embryo research than from a termination, via establishment of therapies preventing defects, so why is embryo research stopped so much earlier? It is surely more ethical to use abandoned IVF embryos for beneficial research (which could aid embryo survival in the long run) than to dispose of them.

Regardless of any extension, we need more clarity in how the Act applies to the new research emerging. The HFE Act 2008 does somewhat cover SC-constructed embryoids as it does not specify embryos made by fertilisation, but there is discrepancy as to whether embryoids resemble embryos sufficiently to be classified legally as an 'embryo'. Likewise, it is undetermined how bioprinting fits into current law because bioprinted embryoids could potentially develop through neurulation, but bypass the primitive streak stage, by patterning the germ layers with signalling centres [27]. The ISSCR released revised guidelines for SC research in 2016, proposing that 'organized embryo-like cellular structure[s] with human organismal potential' [28] should also be prohibited if they breach the 14-day rule. However, this presents an issue: the '14-day paradox' [29]. We will not know if embryoids have 'organismal potential' without culturing them past the point when this potential would be obvious (14 days), so this ISSCR limit requires adaptation.

The Warnock Report emphasised avoiding research on embryos with 'sentience' [2]. Thus, I propose the new guidelines should centre here, setting the limit at the stage where the embryo/embryoid can perceive the environment and feel pain, keeping moral implications at the heart of decisions. Although practically difficult to enforce perhaps, this is necessary because a stage limit cannot hold strong now bioprinting could bypass stages. Additionally, Aach argued that 'days since fertilisation' limits are insufficient for embryoids not formed by fertilisation [30]. Determining sentience is not obvious, yet we need a strict, clear boundary to reassure the public. Therefore, a more objective test, such as immunohistochemistry using antibodies against known early neural markers, could be developed.

Because non-synthetic embryos cannot bypass developmental stages, there could be a different, more objective limit for these, such as 30-34 days, when differentiated neurons and synapses form. Additionally, material from aborted fetuses becomes available at 28 days so *in vitro* culture is less crucial beyond then [12]. However, this diversity in rules might cause a lack of confidence in tight regulations so there must be careful consideration.

Conclusion

The technologies discussed provide great potential to be beneficial not just in advancing scientific discovery but in true clinical benefit. We could enhance regenerative medicine and IVF, reduce miscarriage, and treat birth defects. Although the Act has been integral to embryo research so far, technologies have vastly expanded recently and now the regulation requires careful re-planning. Aach states: "All great scientific advances have a way of exposing the imprecision of common concepts and forcing people to rethink them" [31].

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