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Surgical Model of in Vivo Culture of Stem Cells by Implantation of Fetal Organs into Adult Animals

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Abstract

As far as both alternative and complementary solutions to adult organ transplantation are presently required and stem cell implantation are more and more developed and applied in clinics, we propose the use of foetal organ implantation as a model for in vivo study of ectopic foetal growth and graft / host participation in this process.

In >650 experiments on rats and mice different sites of foetal organ implantation were proposed. Physiological (electric activity, motor and secretion activity) and morphological (per illumination, optic and electron microscopy) methods were used for implant development evaluation during up to 12 months

Results have shown that after a “dedifferentiation” phase foetal organ implants can grow following ontogenesis pattern but – at least in our experimental conditions - some of them are not able to re organize as a whole functional adult organ. The theoretical interest and application perspectives of this surgical model are discussed.

Key Words: Foetal organ development, precursor cells, stem cells, regenerative medicine, reparative surgery, surgical models.

Abbreviations: IGF-1: Insulin-like growth factor; PTH: parathyroid hormone; M: mean value; MRI: magnetic resonance investigation; PTHrP: parathyroid hormone receptor protein; SD: standard deviation.

Introduction

Taking into account the problems of adult organ transplantation (graft procurement, life lasting post transplantation treatment and its complications [1-7]) other solutions are presently envisaged in reparative /regeneration medicine and surgery. Studies about stem cell use have now extended to industrial production and clinical applications [8-13], though some questions remained open, and biology of ectopic development of the grafted tissues is still weakly investigated. Human organoids are also produced and investigated [14-16].

The aims of the present work was to try organ in vivo cultures by means of foetal organ implantations into adult animals (part I) and their evaluation for repairing experimental lesions (part II).

Material and Investigation Methods

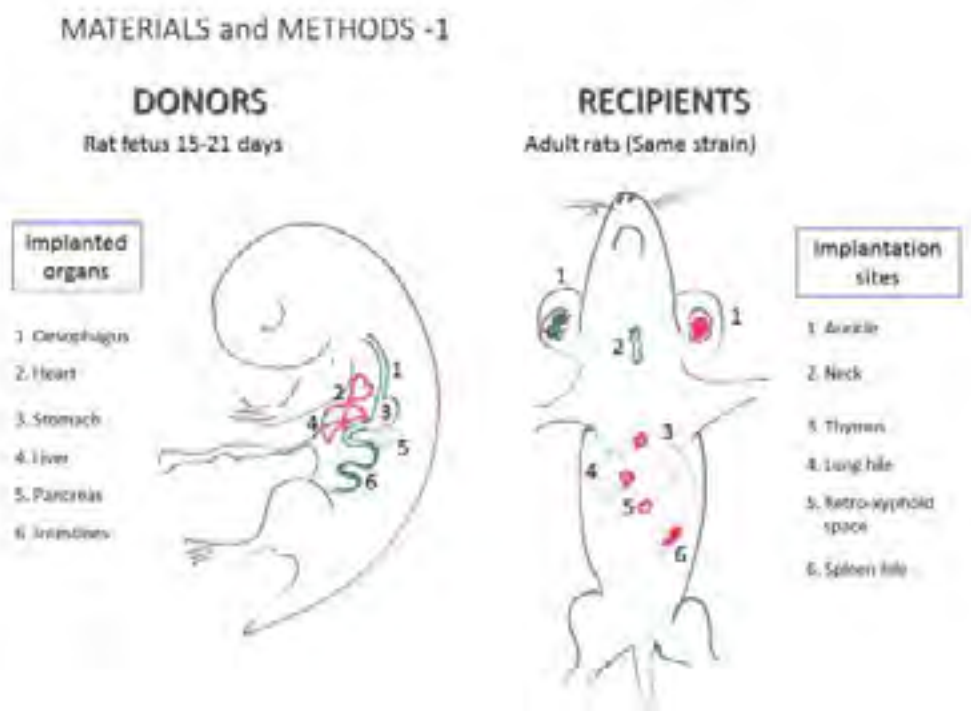
Experiments were carried on more than 650 adult Wistar and Fischer rats, and C57Bl and BALBc mice both sexes, aged 2-6 months, according to Bioethics rules, and allowed by local Ethics Committee. The complete enumeration of the experimental series is presented in table 1. These experiments were performed in syngeneic system of transplantation.

Table 1: Number of animals and experimental series

Series (implants)	Implantation site	Animal nb (recipients)	Observation delay
Fetal esophagus	Ear Neck	10 30	6 months 9 months
Fetal stomach, intestine	Ear Neck	>100 23	12 months 6 months
Fetal umbilical cord	Ear	20	4 months
Fetal heart (FHI)	Ear Thorax	30 40	14 months 12 months
Fetal liver	Ear Spleen hile	29 21	12 months
Fetal pancreas (FPI)	Ear Spleen hile	104 14	9 months
Esophagus lesion + GFEI	Neck	20 + 5	13 months
Heart lesion Idem + FHI	Thorax	20 40	6 months
DM (SD.or APDD) DM + FPI	Ear, abdomen	20 + 37 34 + 38	6 months

GFEI: Grown foetal oesophagus implant

Figure 1: Schema of the different variants of foetal organ implantation provided



The schema of different operations is presented on **Fig. 1**.

Donors were foetuses aged 14-20 days in utero. Under Fluorotane/ Isofurane 4% pre anaesthesia, and after intra peritoneal injection of Nembutal © 0.75 mg/ kg and of Temgesic © 0.5% 0.2 ml as main anaesthesia, laparotomy of the gravid female was provided and the foetuses were extracted, the target organ was isolated and placed in a cup with saline at ambient temperature.

Recipients were anaesthetized in the same way, after what a subcutaneous pouch was created after 2-3 mm skin cut and blunt separation from the subcutaneous layer of the ear pavilion. A piece of oesophagus, stomach, intestine, liver, pancreas (volume 2-4mm^3) or the whole heart was introduced into the subcutaneous pouch. The skin wound was closed either by 8.0 Ethilon stich or by clay (Nobecutane ©).

When thorax site was used, after complementary subcutaneous injection of Atropin 1% 0.2ml, intubation and start of artificial ventilation (UNO – Intermed – Penton, Sigma Delta, Netherland), a classical sternotomy was performed. The implant was either dropped into the cavity, or introduced under the pleura of the lung hilum, or under the thymus capsule, or fixed on the heart apex [17]. After what the thorax was sutured by stiches layer after layer: sternum (Vicryl 2.0), muscles and skin (Vicryl 4.0)

In the case of abdominal implantation, laparotomy was performed and the implant was either dropped into the cavity or placed under the visceral peritoneum in the region of caecum or spleen hilum. The abdominal wall was sutured in 2 layers: peritoneum and muscles by a continuous catgut or Vicryl 4.0, the skin - by Vicryl 4.0 stiches.

Models of pathology were also used to test the possibilities of correction by foetal organ implantation. They included diabetes mellitus induced by Streptozotocin© intra peritoneal injection (75 mg/ kg) or by protein deprivation, oval or circular resection of the oesophagus and thermic lesion of the heart apex, which are described other where [18-21].

After surgery the animals were placed into individual cages during the whole observation period in order to avoid competitive situations able to influence the survival and general condition of the animals.

The investigation methods included:

Clinical observation, BW evaluation (once/week during the 1st month, further once/month),

Physiological methods: depending on concerned organ – ECG (Mouse Monitor, UNO USA-NDL), radiography (standard clinics apparatus, General Electrics Digitalized, USA), ultrasound (Siemens MSH, with a 14, L5 SP probe), MRI (Philips, 1.5 Tesla), pH-measure.

Biochemical methods - analysis of blood and urines by strips for glucose and protein determination, determination of IGF1 in recipient serum by Elisa method). Also, some foetal intestine implants were accurately separated from surrounding tissues, frozen in liquid nitrogen and kept at –80°C till analysis. PTHrP mRNA and its receptor mRNA were quantified by RT- PCR in real time. Control investigations were provided on foetal intestines before grafting and intestines of rats at the same delays after birth as after implantation . Intact animals and animals after sham operations were also used for control.

For every observation delay the number of investigations was no less than 4-6, M and SD were calculated and results compared to control – intact animals or sham operation using Student Td versus Tst.

Morphological methods: per illumination and graft dimension measures, optic (including immuno-histochemistry for glucagon, somatostatin and insulin determination in pancreas implants) and electronic microscopy on biopsy material, fixed in Formaldehyde 12% or glutaraldehyde, embedded into paraffin or araldite. Slices of 4mcm thickness were stained mainly by haematoxylin eosin. For statistics evaluations variation methods and Student criterion were used.

The observation delays ran from day 0 to 600.

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Results

They concern mainly the experiments devoted to implantation of foetal organs in intact adults (feasibility study).

Experiments have shown that some conditions are required to ensure the success of the operation:

1. Short delay between isolation and implantation of the foetal organ < 50 min (the shortest is the best)– (table 2).
2. A well vascularized bed/site for implantation.
3. Absence of immunological conflict, i.e. syngeneic grafting (allogeneic foetal grafts were rejected in the same classic way as adult one).

Table 2: Survival of the syngeneic foetal heart implant (survival number/total implants) depending on « ischemia » delay

Ischaemia (min)	*2 weeks*	*4 weeks*
< 20 min	31/31 = 100%	29/29 = 100%
30-50 min	53/53 = 100%	44/47 = 91%
50-60 min	15/16 = 94%	8/12 = 66%
= or > 60 min	3/5 = 60%	2/4 = 50%
Total implants	102/108	83/92

NB On every animal both ears were used.

Implantation of foetal organs by dropping them into the thorax or the abdominal cavity was a failure.

Implantation under thymus, salivary gland capsule as well as under the spleen hilum or the ileocecal angle visceral peritoneum, was successful in 50-75% of the cases.

Implantation into an ear pavilion subcutaneous pouch presented the possibilities of visualization of the implant evolution, of the measure of the graft dimensions and vascularization features (**Fig 2**), as well as biopsies procurement.

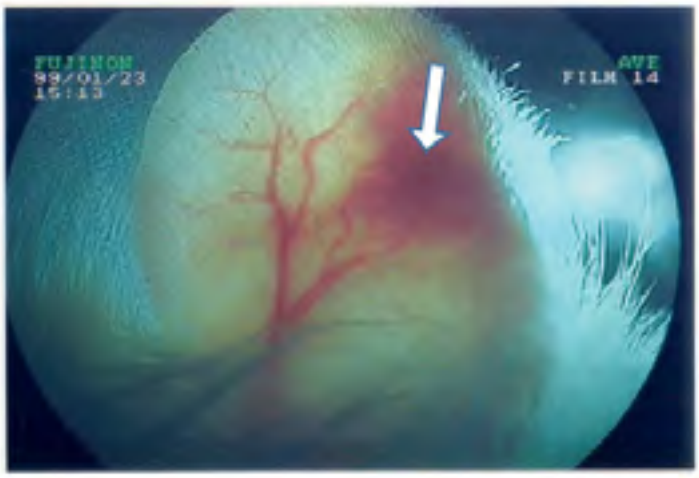

Figure 2: Per-illumination picture of a foetal heart (arrow) and its vascularization within 4 months after implantation at the ear site.

During the first days independently on the implanted foetal organ concerned, the evolution of the implant was the same – the specific organ structure was replaced by a kind of infarcts with cell apoptosis and necrosis and the predominance of undifferentiated cells (**Fig 3**).

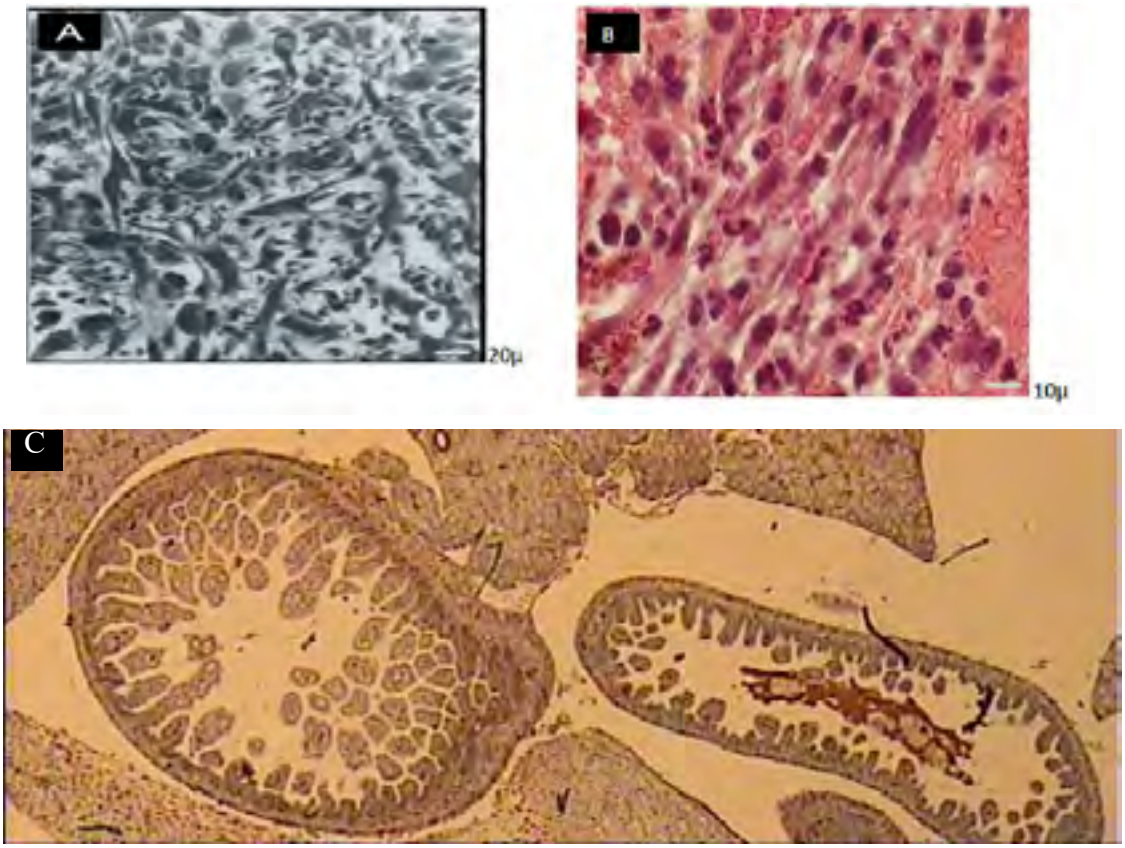

Figure 3: Histology of foetal intestine (A. left) and foetal heart (B. right) at day 4 after implantation (Hematoxilin eosin). The structure and specific cells of both organs have disappeared.For comparison (C) – histology of a foetal intestine just before implantation (haematoxylin eosin, x2,5).

At these observation delays it was even difficult to identify the nature of the implant.

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Penetration of recipient capillaries into the graft and their connection with the graft lacunas were observed about 5-7 days after operation.

During the next weeks, differentiation of tissues was realized according to ontogenetic pattern specific to the implanted organ (Fig.4).

Figure 4: Histology of implants at different delays after operation. Reconstitution of practically normal adult organ within 2-4 months after implantation:

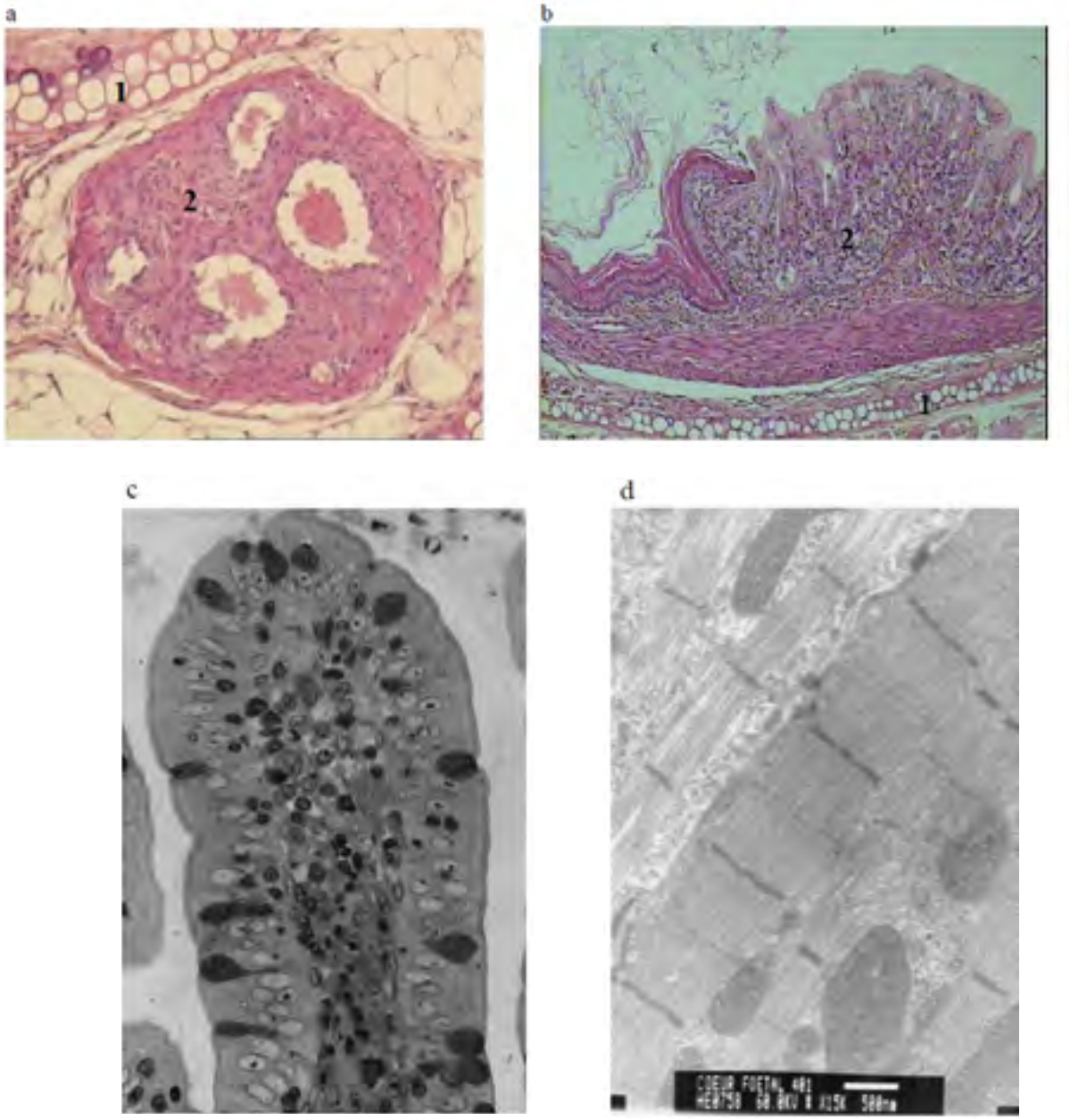
a. Heart,

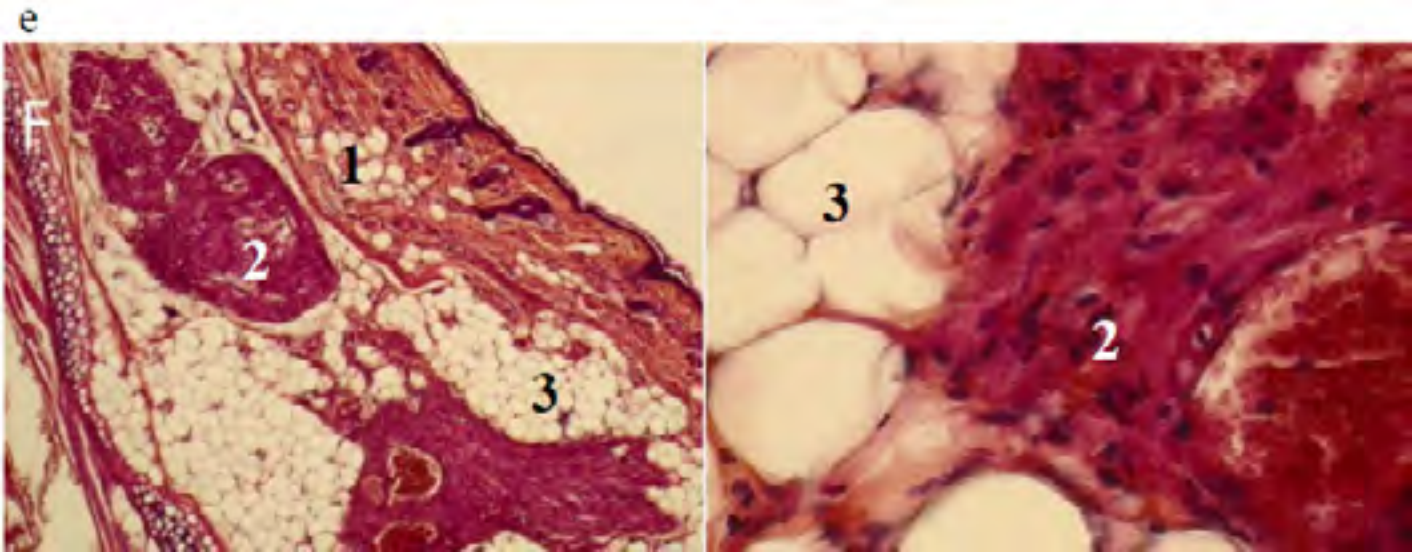
b. Stomach with oesophagus at the ear site or Cardia zone of an oesophagus-stomach implant. 1. cartilage, 2 implant, 3 adipose tissue (haematoxylin eosin).

c Intestine implant electron microscopy with evidence of chylomicron absorption at month 4.

d. heart implant electron microscopy at month 4 with evidence of normal intracellular structure.

e. Aging of the foetal heart implant 11 months after operation: fibrosis and adipose replacement of cardiomyocytes.





As an exception teratomas were observed in 3 cases, when very young donors (BW < 1g) were used [22]*.

According to first investigations IGF-1 and PTHrP levels were slightly elevated at day 7-14 post operation. PTHrP was high in normal fetal intestines and slowly decreased after birth. In the case of ectopic development a slight decrease was noted during the first days, that corresponded to the necrosis and apoptosis noted in the graft. At days 7-9 a pike of activity was expressed, corresponding to the beginning of the re-formation of the grafted intestine. What the receptor was concerned, the expression has shown a trend to decrease. During the further 4-6 months, only IGF-1 values were significantly elevated reaching 200% of their initial and control levels (**table 3**).

Table 3: First results of IGF-1 determination in recipient sera (ng/ml)

Observation delay	Foetal intestine & stomach	Foetal heart	Foetal pancreas	Significance (vs control)
Control (intact)	460 ± 31	460 ± 31	460 ±31	
Day 4	599 ± 130	589 ± 37 *	689 ±200	*p<0.01
Day 9-14	1120 ± 200*	nm	713 ± 58*	*p<0.01
Month 2-3	922 ± 85*	nm	672 ± 25*	*p<0.01
Month 6	624 ± 56*	608 ± 80*	498 ± 11	*p<0.01

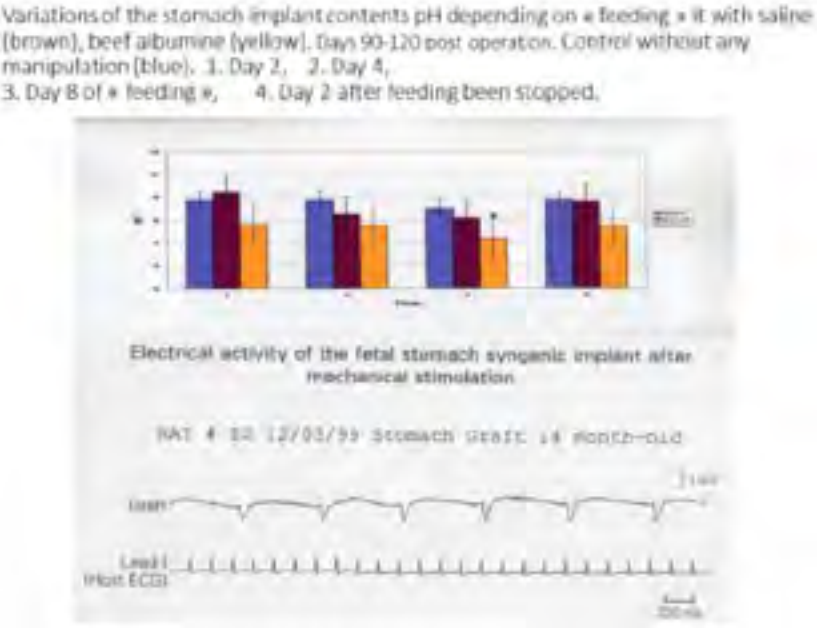
The grown implants of foetal heart, intestine, stomach, and oesophagus have proved to be functional: motility, secretion for intestine and stomach, presence of a cardiac rhythm and blood flow for the heart were detected (**Fig.5**).As to liver and pancreas, adult structures were obtained but not an organ functioning as a whole. So liver has shown bile system and hepatocyte columns with sinusoids, but no sign of bile elaboration. The grown foetal pancreas developed pancreatic tubes, sometimes acinar formations and the presence of endocrine cells insulin, glucagon and somatostatin stained by histochemical methods but no pancreatic organization. Nevertheless foetal pancreatic implants have shown functional activity (see [18, 19]).

In late delays (8-11 months) the implants have shown signs of aging: fibrosis or adipose degeneration (see Fig.4d).

*6 cases, i.e. <1% if the whole material, that is more than 650 operations, is considered.

Figure 5: Evidence of functional capacities of stomach and heart grown implant (site ear).
A. Acid secretion of the implant after protein stimulation of its mucosa.
B. Electric activity of the fetal gastric implant.
C. Magnetic resonance investigation of the fetal heart implanted into the ear pavilion at month 4. Successive modifications of the form and position of the implant cavities testimony the probable presence of contractions and blood flow.
D. Electric activity record (electrodes placed in the ear pavilion on both sides of the implant of foetal heart 4 months after operation): frequency and wave form of the graft ECG are influenced by both adrenalin injection and by vagus stimulation of the host.

Figure 5. A.B.



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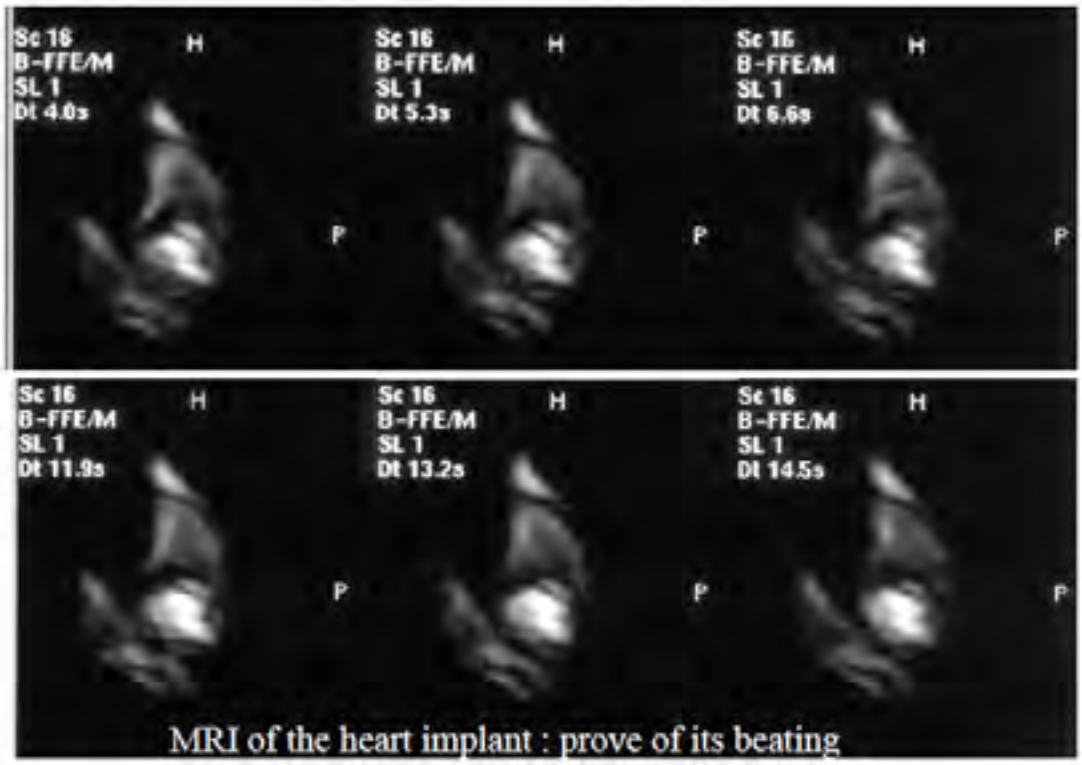

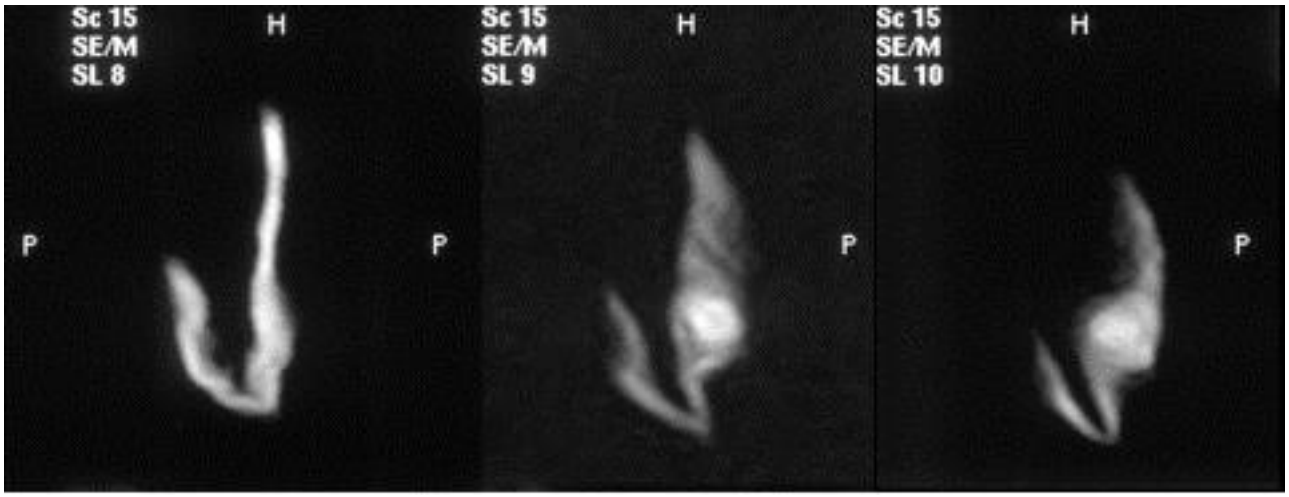
Sc 15
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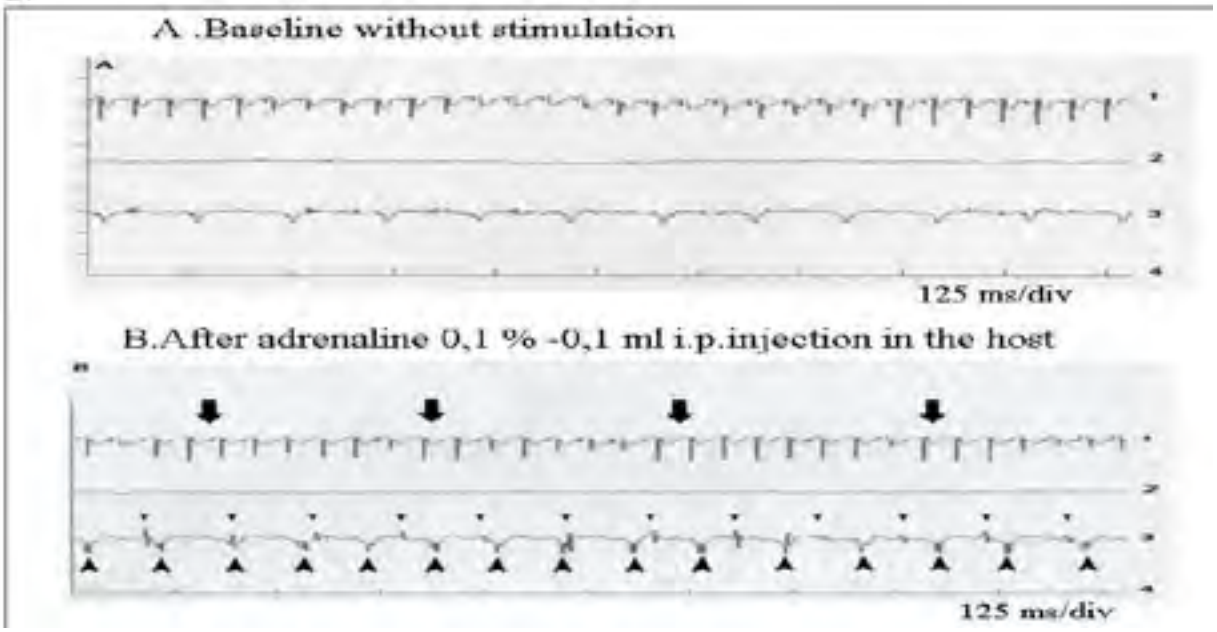
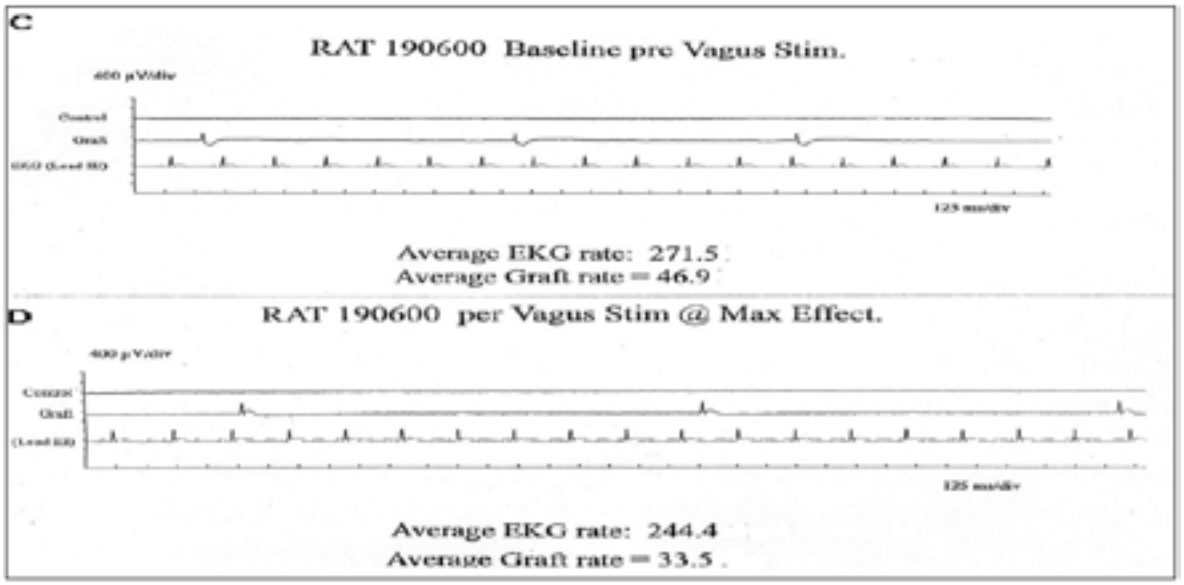

A .Baseline without stimulation
125 ms/div
B.After adrenaline 0,1 % -0,1 ml i.p.injection in the host
125 ms/div


C
RAT 190600 Baseline pre Vagus Stim.
Average EKG rate: 271.5
Average Graft rate = 46.9
D
RAT 190600 per Vagus Stim @ Max Effect.
Average EKG rate: 244.4
Average Graft rate = 33.5

Discussion

The comparison of the different surgical models of foetal organ implantation used in our experiments has shown that the best results (up to 95-100% success) were obtained in the case when the warm ischemia duration did not excess 50 min, and the shortest was the best. We did not use cooling of the procured organs, as far as preliminary tests were negative: cold altered foetal cell condition. Important too that the implantation site was well vascularized: under gland capsules or visceral peritoneum, or within the ear pavilion. Localizations in the spleen or liver or kidney superficial parenchyma are well known and also satisfy to the same criteria [23-29]. The subcutaneous ear pavilion seems to be an original implantation site and has shown different advantages: simple, easy to perform and not very traumatic operation, well tolerated by animals, allowing visual observation, measures, biochemical and physiological investigations of the implant, procurement of biopsy material. The only inconvenient was the limited volume of the implant possible growth. The neck implantation site ensured place enough from even enormous growth of intestine or stomach adult-like cysts. In our experiment we did not ensure the exit of secreted mass, though it is possible and spontaneously occurred in some cases (see also [23]).

The evolution of the implanted foetal organ has shown different phases. First a "destructuration-dedifferentiation" phase during the first week after operation. From day 2 to day 5-7 the observed picture may be interpreted either as regression, dedifferentiation of the organ structures, or as the result of mature cells necrosis whereas weakly differentiated ones survived and gave a new development after a while. Unfortunately up to now we had not had the possibility to perform the necessary tests with stem cell marking or determination of host/graft cells into the mixture of lymphocytes, fibroblasts and other not identified cells. Interesting that at this moment it was difficult to identify the very origin of the graft (see fig.3). Phase 2 is characterized by differentiation of the "magma" cells which formed an adult like organ at the end of the first post implantation month. During phase 3 the structure stabilized and function developed, maintaining during several months. Phase 4 was characterized by aging phenomena, morphological and functional degradation.

Nevertheless, during the second phase of re-differentiation, the reconstitution and development of the implanted organ was specific and never deviated from the ontogenetic pattern. Only when we tried umbilical cord implantation, we obtained the growth of an intestine but only when the foetal half of the cord was implanted. That may be explained by the presence in that half of allantois, coming from endoderm and primary intestinal tube. These observations mean that re differentiation probably occurs from precursor cells already organ determined. This also signifies that the first phase is worthwhile further investigations not only for cell identification, but also in order to influence both their differentiation and development.

It is necessary to note that we observed the growth of teratoma in 6 cases (out of more than 650 operations), including 2 cases of malignancy. This was previously discussed [22], but it is important to underline that it always occurred when the donor was very young (<1g BW, < 12 day in utero age) and so could contain embryonic multipotent stem cells, and when the foetal organ was implanted into a zone of the adult organism where stem cells could be easily mobilized. The absence or small quantity of pluripotent stem cells in the implant procured from donors in the last third of gravidity, might also be a warrant of its correct, without tumour deviance, development.

We have also observed that only hollow organs with intramural nervous system or conduction system and motility as oesophagus, stomach, intestines and heart, were reconstituted as whole organs with organized and effective functional activity. As to liver and pancreas, their constitutive elements were present, even functional (insulin secretion able to influence diabetes evolution in rats after Streptozotocin injection [18, 19], but coordinated activity of the whole was absent (no sign of exocrine secretion both in pancreas and liver). These organs are innervated differently, without a "peripheral brain" within them. Is this the cause of the different evolution of the implant ? Besides studies about re innervation of stem cell, islet cell implants were started but without strong conclusions [30, 31].

Interesting also to observe that growth factors values have been elevated in the graft tissue homogenate and in the recipient blood after operation. Both PTHrP expression and significant growth of IGF1 in the recipient serum were registered beginning from the second week after operation (beginning of the 2d phase of the graft evolution) when the first connexions between the host vascular web and the graft are realized and the graft began to form again. Later the PTHrP evolution was less sure, but the IGF1 increase reached up to 200% and more than its initial value and lasted all through the second and third phases, during which the implant morphologically and functionally looked like an adult like organ.

Linked with the previous observations and reflexions the question of the respective role of donor and recipient in the implant development must arise. Why the host IGF1 levels remained significantly high during months that corresponds to the 2d and 3d phases of the foetal intestine and heart implant growth ? They normalized later at the 4th phase when aging and fibrous or adipose degeneration of the graft developed. What is the origin of the phenomenon and its mechanisms ? Is it a cause or a consequence of the implantation and development of the graft ? What are the adult organism limits of stem cell mobilization for tissue and organ repair ? How to influence these phenomena and avoid deviances like tumour formation ?

Presently this work cannot give any answer: deeper analysis and complementary investigations are needed. No answer too to the question of the origin of the grown implant cells and to the possibility of their integration to the host organism (that is especially important for heart repair). Implants of digestive organs were morphologically distinct from recipient tissues except when implantation was performed into a lesion site: oesophagus defect. So were foetal heart implants: when foetal heart implantation was performed in the site of heart apex injury, in last delays tight junction with recipient tissues was often observed. But we have no liable proofs of any degree of the implant integration. For that a liable marking of either donor cells or recipient ones is necessary. It

ought to be the aim of further studies (ours or other researchers'). Nevertheless, the results observed in our trials with foetal oesophagus-stomach- intestine use for oesophagus defect repair or with implantation of foetal heart on the site of a thermic injury of the heart apex, were positive. Detailed description of the operations and discussion of their results will be the subject of separate further communications (part II).

Indeed, the described operations are only surgical models, may be proposals of surgical models. They are low cost and simple execution. They are not exclusive: a lot of foetal organs was not tested here such as brain, kidney, lung, and other implantation sites are also possible, many of them having been mentioned in literature [23-38]. Our studies have many common features with organoid creation studies, except that the last are started in vitro and their growth can be obtained from other organs of the same embryonic sheet (for instance intestine crypt cells can give growth to pancreas) by experimental monitoring means [32].

Surgical models of different stem cell category involvement during foetal organ implantation open wide possibilities to experiment in vivo new methods of influencing (boosting, slowing or modifying) the foetal organ graft development. They might be an intermediary step between in vitro studies and clinical applications in the field of tolerance research, for instance. So we hope that this work will be useful for further researches and experimentations in the field of reparative surgery and regenerative medicine.

Conclusion

1. Surgical implantation of foetal organs with its features of regression followed by ontogenetic development seems to be an interesting model for the IN VIVO study of such phenomena as possible de-differentiation of tissues and managing re-differentiation growth factors, and be useful for developmental biology investigations.
2. The ear site allows delicate dynamic observations with pointed methods.
3. Implantation of foetal organs might also be applied to clinical purposes in relatively short delays, the main problems to resolve (apart ethics) being: a) development of acquired immunological tolerance rather than immunosuppression and b) constitution of foetal tissue/organ banks with in vitro as well as in vivo storage (see [39]).

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