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(54) Titre : MICROENCAPSULATION DE CELLULES VIVANTES DANS DES MEMBRANES D'ALGINATE-POLY-L-LYSINE-ALGINATE A RETICULATION COVALENTE

(54) Title: MICROENCAPSULATION OF LIVING CELLS IN COVALENTLY CROSSLINKED ALGINATE-POLY-L-LYSINE-ALGINATE MEMBRANES

(57) Abrégé/Abstract:

Immunoisolation of cells in semipermeable membranes has been proposed as a means to prevent their immune destruction following transplantation[1, 2]. However, several fundamental issues remain to be addressed before considering clinical application of this method. One such issue is the resistance of microcapsules to chemical and mechanical degradation. Strong microcapsules will obviously increase the durability of the transplant. It is also likely to improve long-term biocompatibility of microcapsules, since a strong pericapsular reaction always develops around broken or damaged capsules[3, 4]. Moreover, the strength and durability of microcapsule membrane are critical issues for the transplantation of virus-transfected bioengineered cells. These virus-transfected bioengineered cells potentially include insulin-producing cells as well as cells producing molecules, which promote islet cell survival/replication/transdifferentiation or insure islet and microcapsule immunoprotection. Finally, considering the limited supply of human islets, the most promising approach to β-cell replacement is the transdifferentiation of stem cells into islets[5] (not necessarily embryonic stem cells, which arise controversy, but stem cells of many other origins, including autologous cells). Note that even autologous cells would require immunoprotection against the recurrence of the autoimmune process. The major concern related to this approach is the risk of malignant transformation of immature stem cells. Microcapsules that can hardly be destroyed in conditions compatible with life would provide a safe method of transplanting stem cell derived cells, bioengineered cells or immortalized cells. Complexation between alginate and polycations such as poly-L-lysine(PLL) is the most widely used method to microencapsulate cells[2]. Microcapsules are constructed by a simple three steps procedure. First, the cells are entrapped in a bead formed by the ionic cross-linking of alginate by a divalent cation such as calcium[6]. The second step comprises coating the alginate beads with a polycation such as PLL, which forms a peripheral membrane ensuring a good control of the molecular weight cut-off and increasing membrane stability. Finally, the third step consists to coat the microcapsules with a dilute alginate solution for insuring biocompatibility. The two latter reactions rely on the ionic interactions between the polyanionic alginate and the polycationic polymer of lysine[7, 8]. In order to improve microcapsule strength, we[9] and others[7, 8, 10, 11], have evaluated the effect on microcapsule strength of modulating intrinsic parameters PLL molecular weight, concentration and incubation time and the mannuronic acid/guluronic acid ratio of alginate. The formation of neutral capsules by the introduction of a new coating agent[12] has also been investigated. Following these experiments, tighter binding between PLL and alginate was obtained. Nevertheless, poly-L-lysine still competes with other positively charged molecules in the environment to bind to the alginate beads. A prolonged incubation in solutions with high concentrations of Ca^{2+} or Sr^{2+} has showed a displacement of the alginate-poly-L-lysine bounds[13, 14]. In addition, Thu et al. have observed the progressive loss of the external sheet of alginate from microcapsules within days or weeks[7]. To prevent this competition with charged molecules in the environment, a new concept has been proposed: the introduction of covalent links into the membrane of the microcapsule[15-17]. Covalent links are known to be more stable than electrostatic interactions. The challenge is that most methods to create or to break a covalent link are incompatible with the survival of living cells. The objective of the present work was to develop and validate a method, compatible with encapsulated cell survival, to create a covalent link between the PLL layer and both alginate from the microcapsule core and from the outer sheet. It would be unlikely that such a microcapsules would be destroyed in conditions found in the living body. Here we show that it is possible to microencapsulate living cells in membranes composed of covalently linked alginate-PLL-alginate membranes, without affecting cell viability. The formation of a covalent link between PLL and alginate from both the alginate core and the outer alginate

(57) Abrégé(suite)/Abstract(continued):
sheet considerably increased microcapsule resistance to chemical and mechanical stress. Standard APA microcapsules dissolved within 45 seconds when they were incubated in an alkaline solution whereas covalently linked APA microcapsules remained unaltered after two years in the same alkaline solution Covalently linked microcapsules were 22 times more resistant than standard microcapsules when they were submitted to a mechanical stress. The process is based on the chemical derivation of poly-L-lysine (PLL) with a photoactivatable heterobifunctional cross-linker, N-5-Azido-2-nitrobenzoyloxysuccinimide (ANB-NOS), which is able to covalently bind PLL to alginate when energized by UVA light illumination[18, 19]. The N-hydroxysuccinimid of the cross-linked was fist covalently linked to PLL in conditions that would damage living cells, but before the cells are involved in the procedure. Then islet cells encapsulated in calcium alginate microcapsules were incubated with the cross-linker derived PLL, then in alginate again to form the outer biocompatible sheet. When the preparation is illuminated with a UVA lamp the photoactivatable residue reacts with the phenyl azide residue on alginate creating a covalent link between PLL and alginate. The latter reaction is not harmful for living cells. All reactions involving the crosslinker-derived PLL were performed in a dark room, until UVA illumination, to prevent activation of the photoactivatable residue. We, herein, present a proof that a covalent link was formed providing considerable improvement of microcapsule resistance to chemical and mechanical degradation. The results of the study showed that the procedure did not modify microcapsule membrane permeability and did not affect in vitro and in vivo encapsulated cell survival.

ABSTRACT (MAX 150 MOTS, PAS DE RÉFÉRENCES)

INTRODUCTION (Nature:500 mots, REFERENCES +++, SOME OVERLAP AVEC ABSTRACT ACCEPTABLE)

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Application number / numéro de demande: 2437250

Figures: 1, 5, figure 2 - microscopic diagram

Pages:

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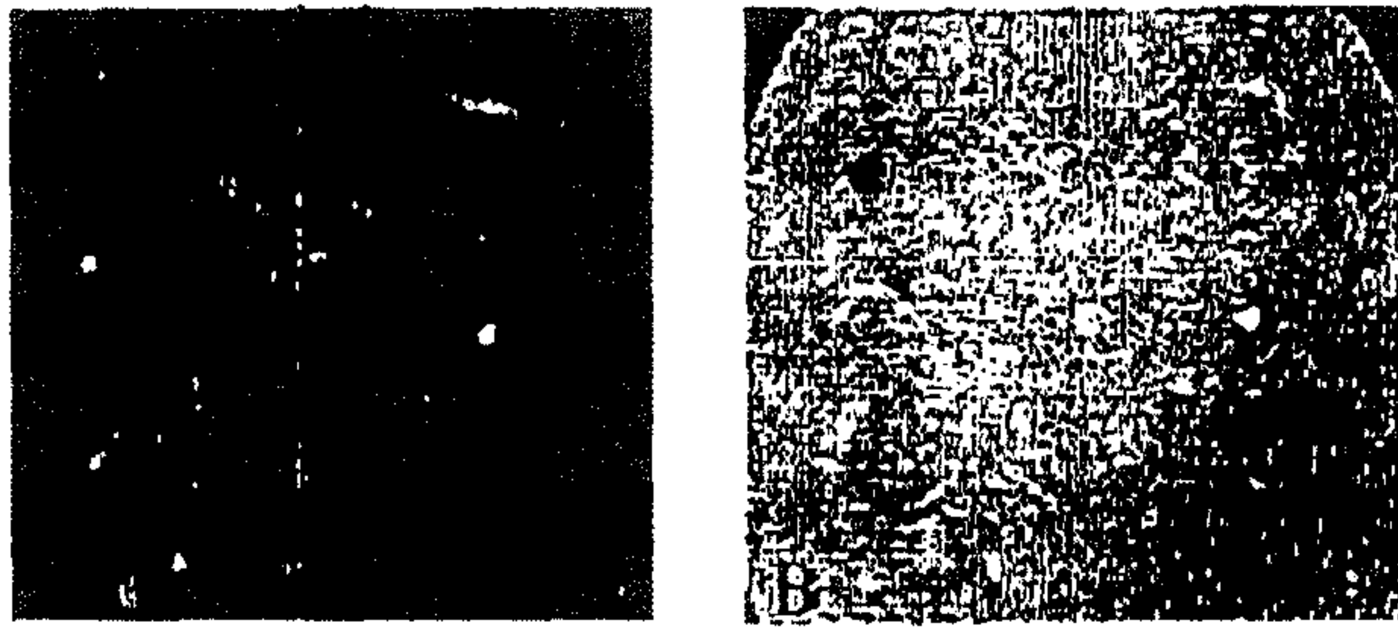


Fig 1: cellules de Sertoli TM4-GFP après 3 semaines de culture dans du milieu sélectif sous microscope à fluorescence A, même cellules sous lumière blanche B.

Résultat: Avec ce protocole on obtient, au bout de 3 semaines, une des populations polyclonales de cellules exprimant le transgène dans des intensités différentes. Environ 80-90% des cellules expriment le transgène

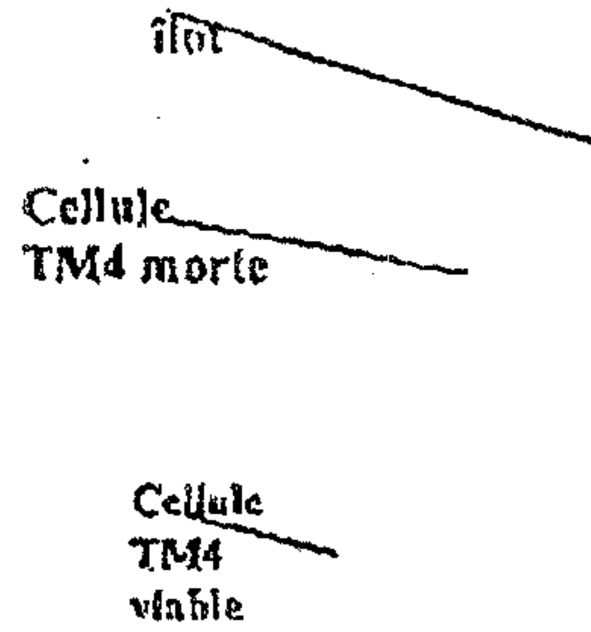
Protocole: Les cellules TM4 à 60% de confluence sont transfectées par un mélange ADN (plasmides pGFP ou pIGFs) : Fugene 6 au ration 1:3. Le contact mélange ADN:Fugéne 6 versus cellules dure 24 heures. Les cellules sont ensuite mises en culture pendant 24 h dans du milieu de culture normal avant d'être cultivées pendant au moins 3 semaines dans du milieu contenant un agent de sélection des cellules transfectées (geneticine G418).

Fig 2: Coencapsulation de 2 millions de cellules de Sertoli TM4-GFP avec 1300 Ieq de rats pour un volume de 650 ul d'Alginate.

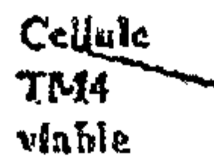
Résultat: Avec ce protocole on obtient des îlots encapsulés avec environ 50 cellules de Sertoli par capsules.

Protocole: Les cellules de Sertoli TM4 sont trypsinées, comptées et la viabilité mesurée au bleu de Coumassie avant d'être lavées deux fois (par 5 ml de saline dans Falcon 15). En parallèle les îlots sont lavés 4 fois (par 1 ml de saline dans Eppendorf). Les cellules TM4 sont mises en suspension dans 500 ul d'alginate qui serviront à mettre en suspension les îlots. On obtient ainsi un mélange homogène d'îlots et de cellules de Sertoli avant le protocole d'encapsulation. Celui-ci est réalisé de la même manière qu'habituellement.

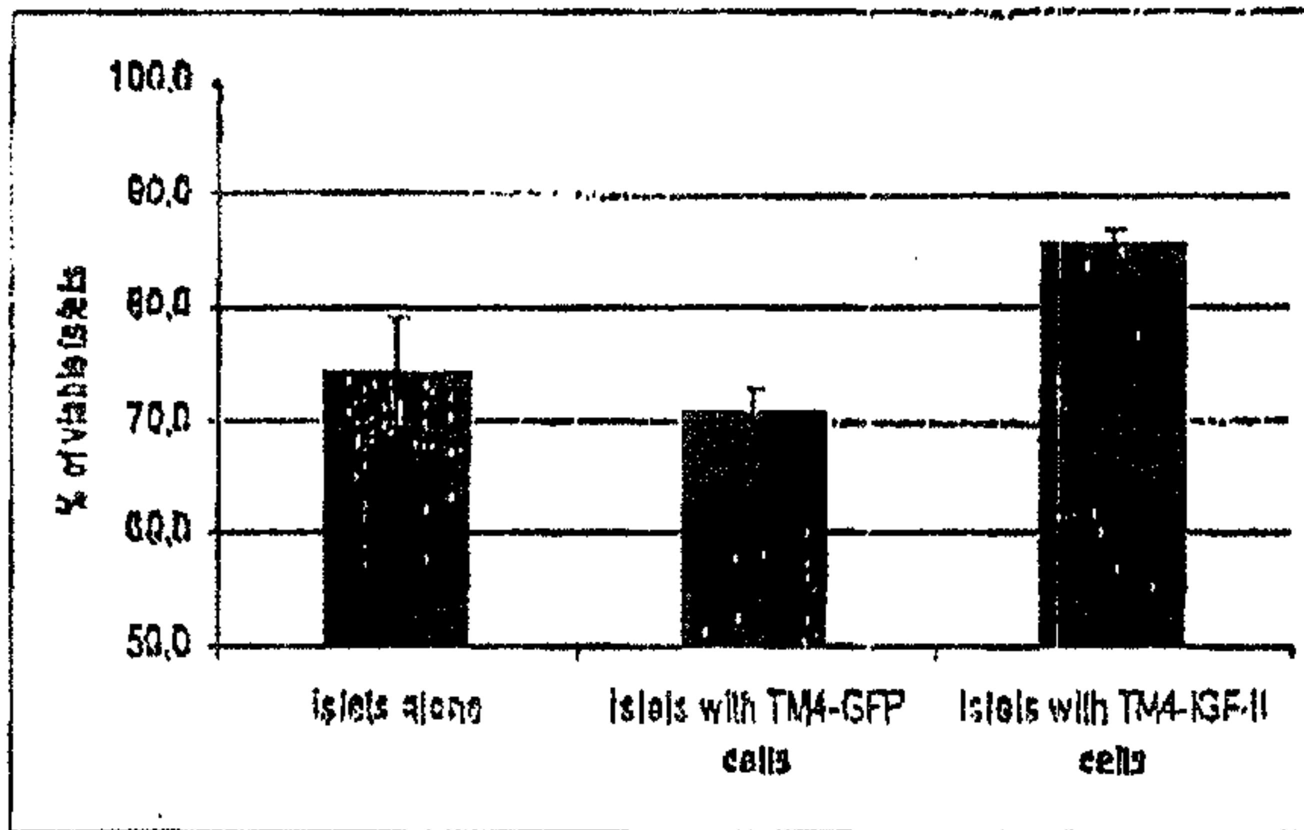
Fig 3: Viabilité des îlots co-encapsulés avec des cellules TM4 exprimant de manière stable GFP ou IGF-II. Test à l'Acridine Orange (AO) et Iodure de propidium(IP), cellules mortes en rouge et cellules vivantes en vert.



Protocole; les capsules contenant les îlots et les cellules sont incubées 10 minutes dans de l'AO et PI avant analyse au microscope à fluorescence.



Résultat: le graphique 1 repsésente la viabilité des îlots coencapsulés avec des cellules TM4-GFP ou TM4-IGF-II ouseul après 3 jours de culture dans du milieu avec serum. Deux observateurs ont mesuré la viabilité sur 150-200 îlots.



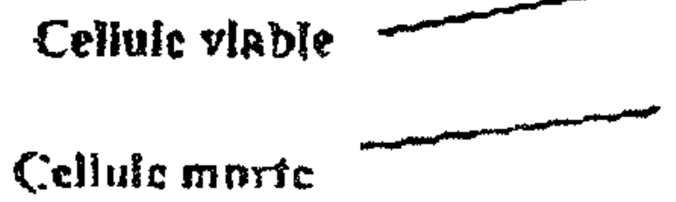


Fig 3: Viabilité des cellules TM4 encapsulés par un test au bleu de Coumassie. On constate que 60 % des cellules sont bleus (mortes) et 30 % sont viables (translusides, blanches)

Protocole: On incube pendant 1 minutes dans un mélange 1:1 milieu bleu de Coumassie les capsules avant de réaliser 4 lavages. L'analyse se fait au microscope.

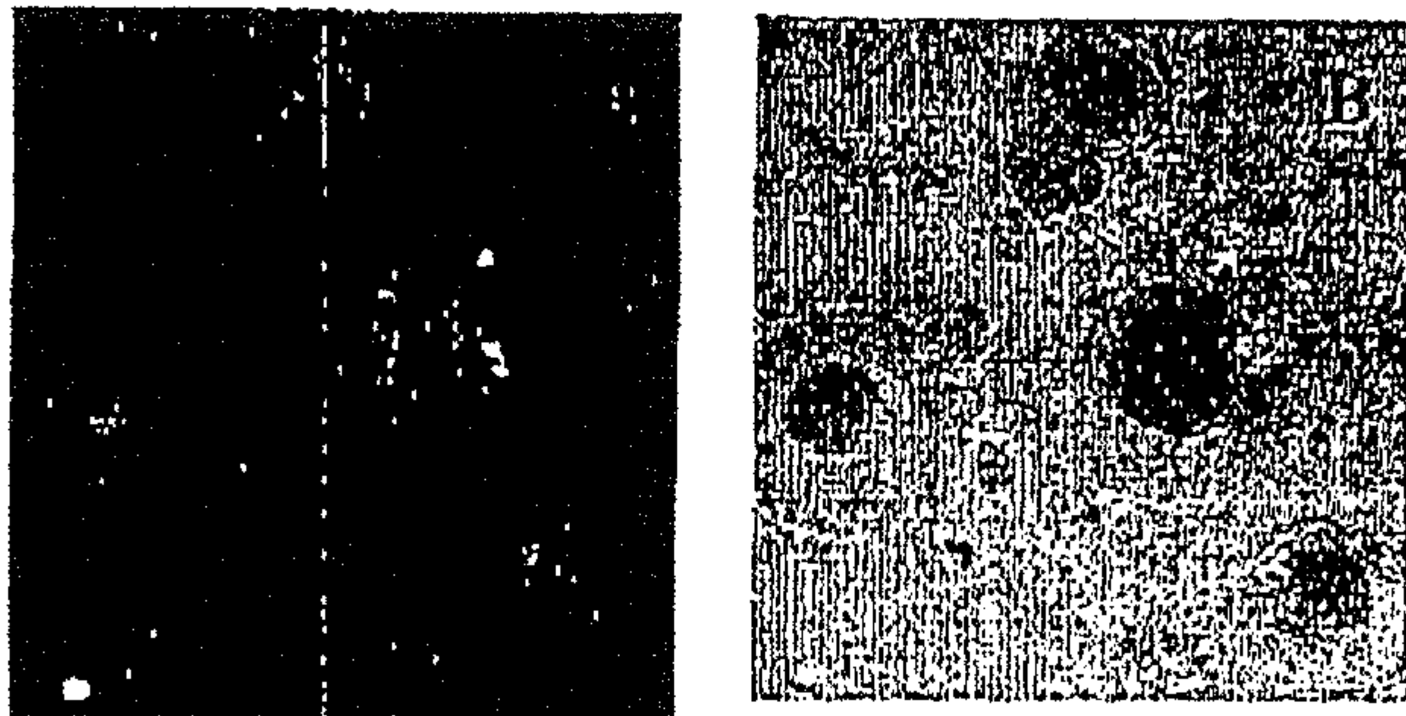


Fig 5: îlots de rats transfectés par pGFP et encapsulés 3 jours après post transfection. A îlots sous fluorescence; B même îlots avec la lumière blanche

Résultats: Le taux de transfection est évalué à 63% des îlots et plus de la moitié des îlots transfectés l'est au moins deux fois (55 îlots transfectés 1X; 42: 2X; 8: 3X; 8: nX et 69 non transfectés).

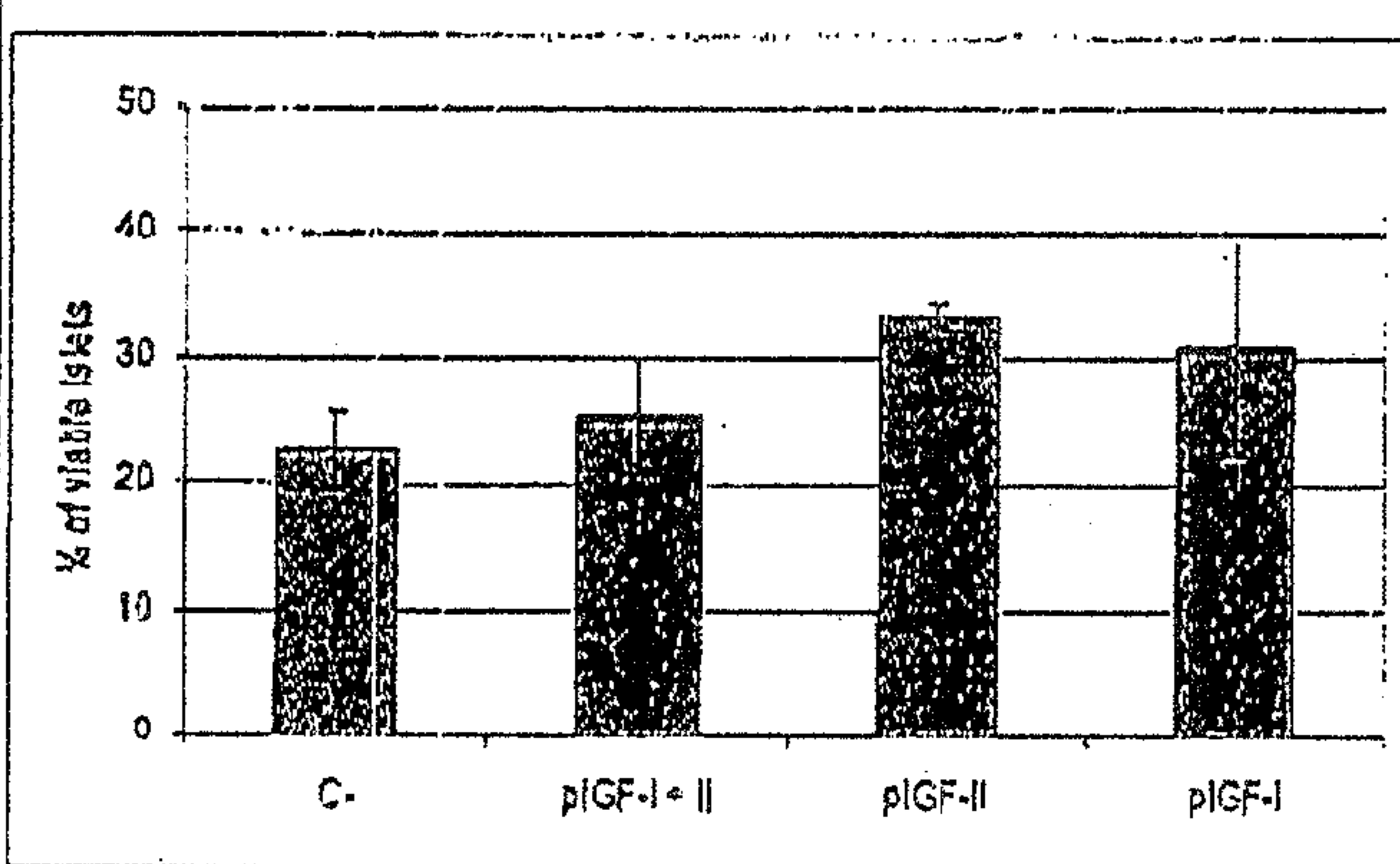


Fig 5: Viabilité d'îlots de rats transfectés (pIGF-I, pIGF-II et pIGF-I + II) ou non (C-) encapsulés et cultivés pendant 6 jours dans du milieu sans sérum. La viabilité est mesurée par IP et AO. L'expérience a été réalisée deux fois et la viabilité mesurée par deux observateurs à chaque fois.

Résultats: Il semblerait qu'une transfection des îlots par pIGF-I et pIGF-II améliore la viabilité des îlots en culture dans du milieu pauvre. Le taux de transfection mesuré en parallèle à la viabilité pour les deux protocoles étaient de 23 et 21 % des îlots, ce qui nous permet de supposer que même un taux de transfection faible protége les îlots et que les premiers jours de cultures sont limitant pour le taux de transfection (les cellules transfectées sont à l'extérieur de l'îlot, se sont les premières cellules à mourir)

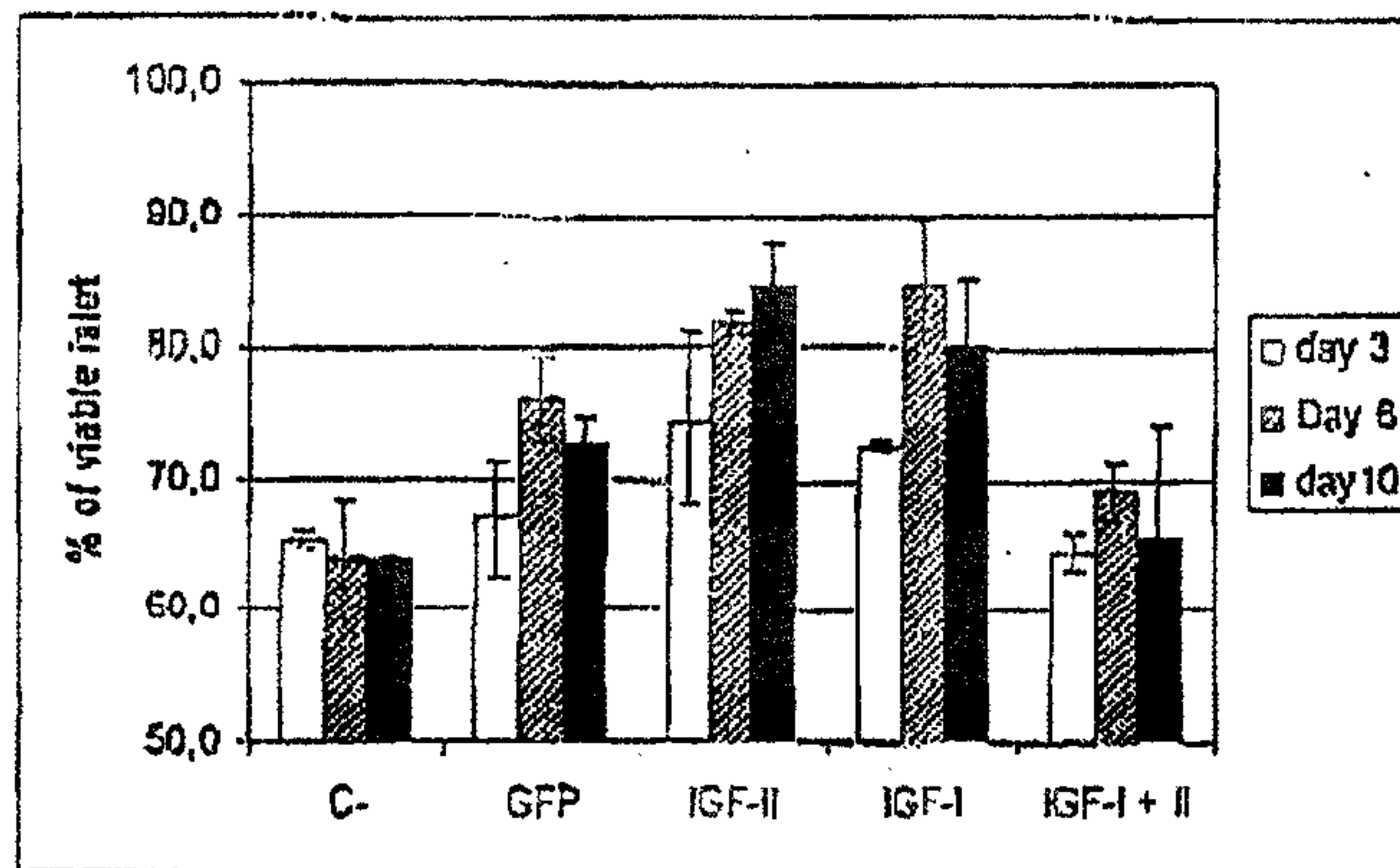
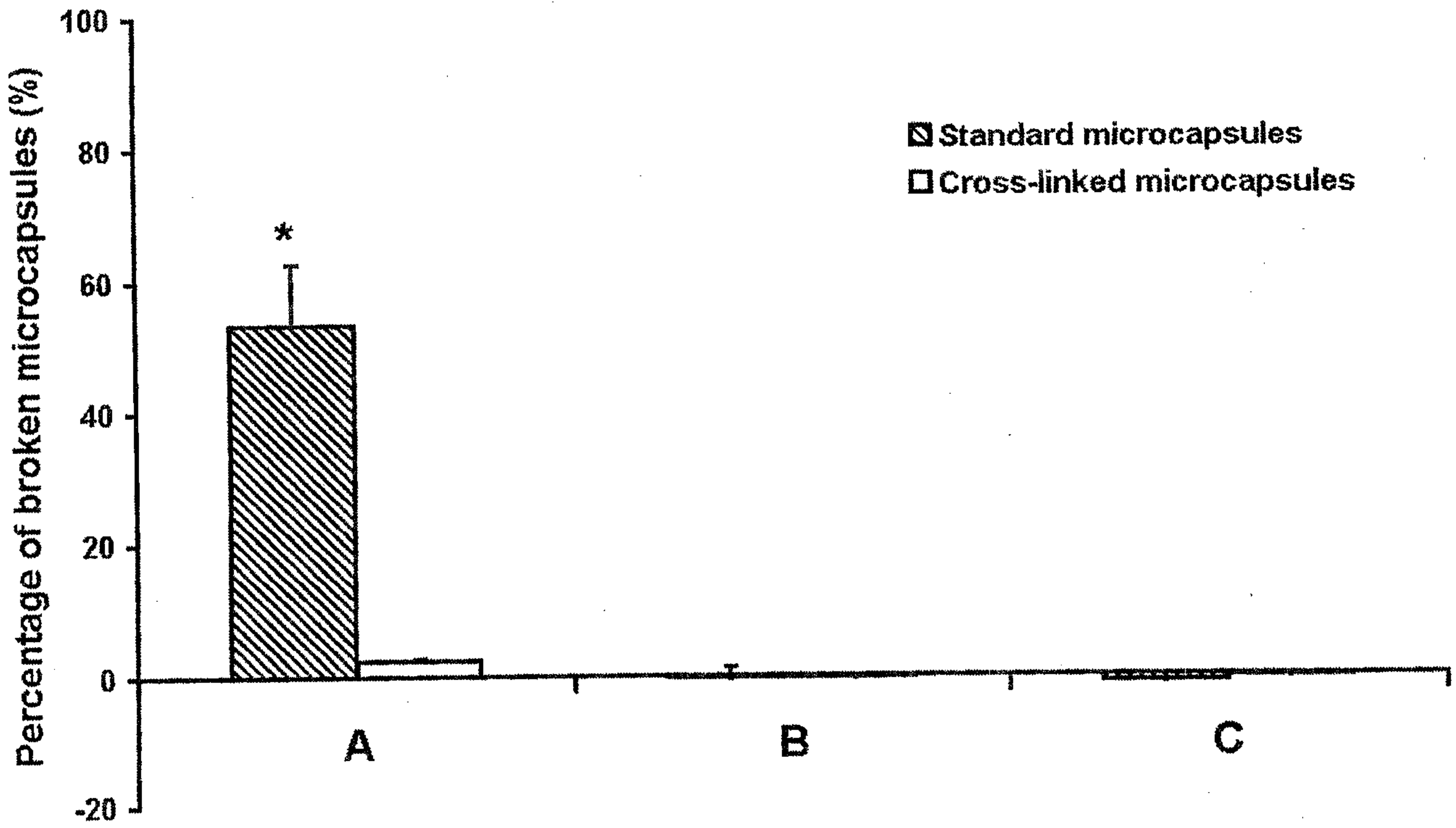
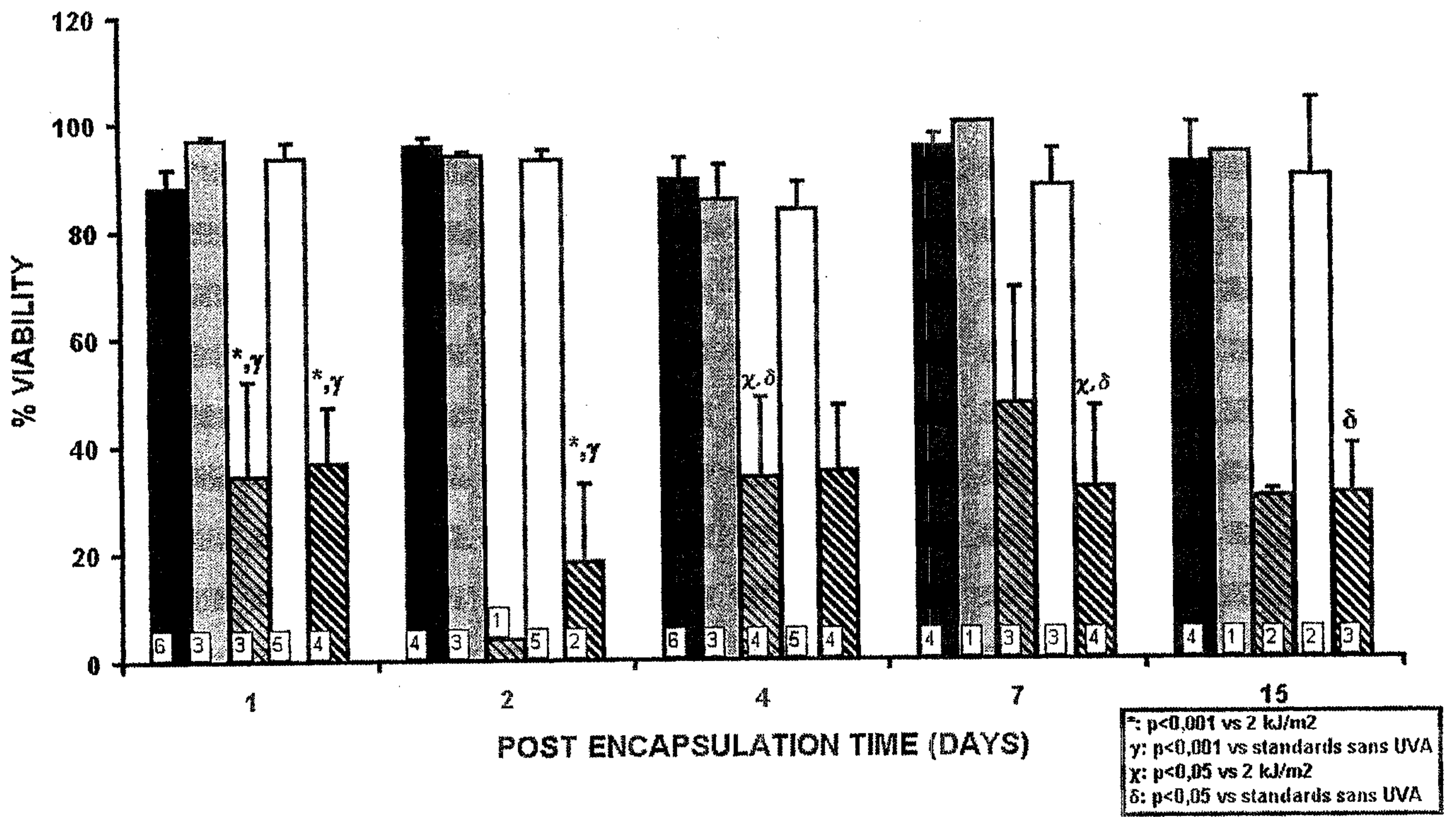


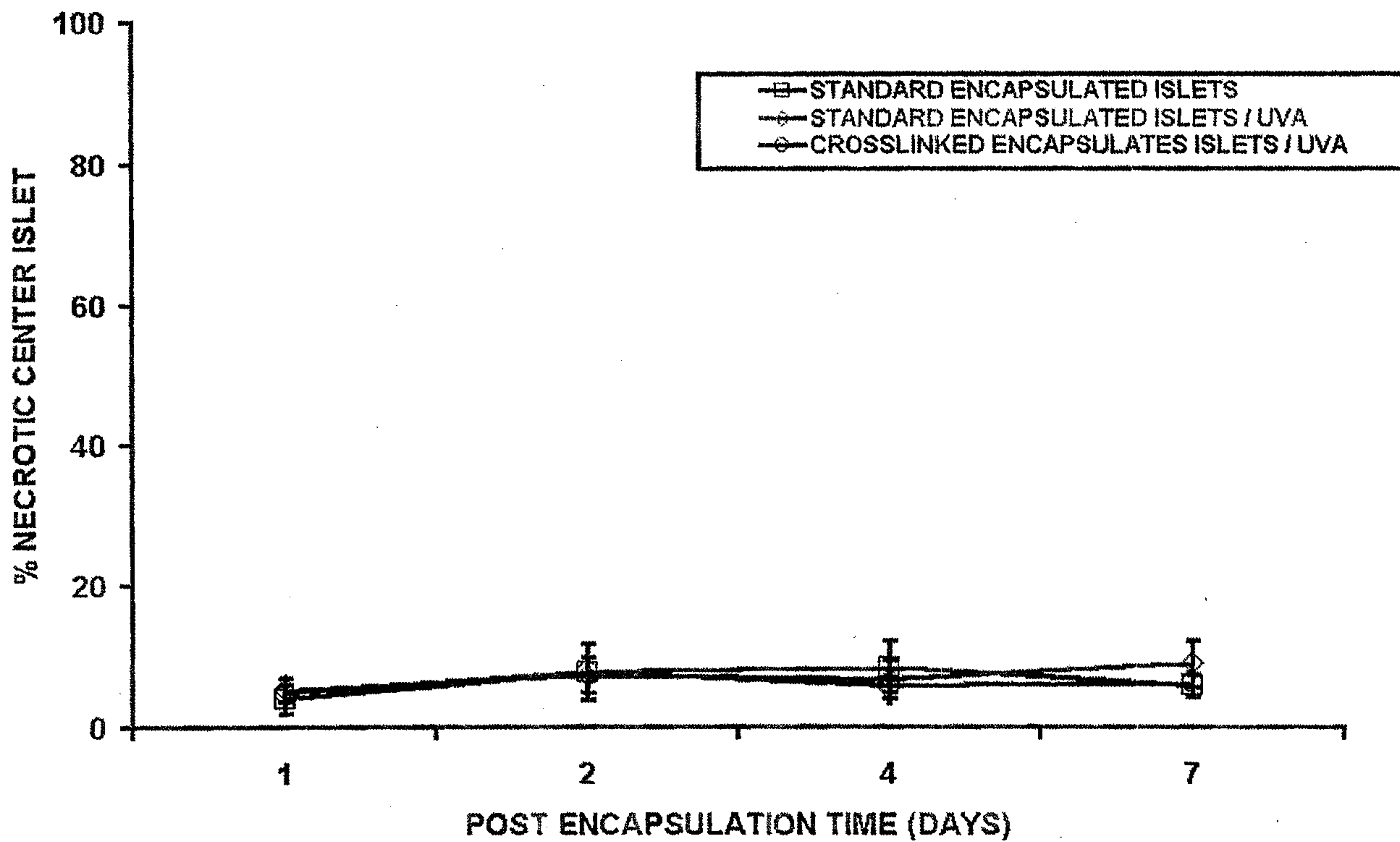
Fig 6: Viabilité d'îlots de rats transfectés (pGFp, pIGF-I, pIGF0II, pIGF-I + II) ou non, encapsulés et cultivés pendant 6 jours dans du milieu avec sérum. Viabilité mesurée par deux observateurs, test à l'IP et AO.

Résultat: Il semblerait que les îlots transfectés par pIGF-II et pIGF-I améliore la viabilité des îlots encapsulés cultivés dans du milieu avec sérum. La taux de transfection à 3 jours de ce protocole était de 63%.



Percentage of broken microcapsules (%)
100
80
60
40
20
0
-20
Standard microcapsules
Cross-linked microcapsules
*
A
B
C

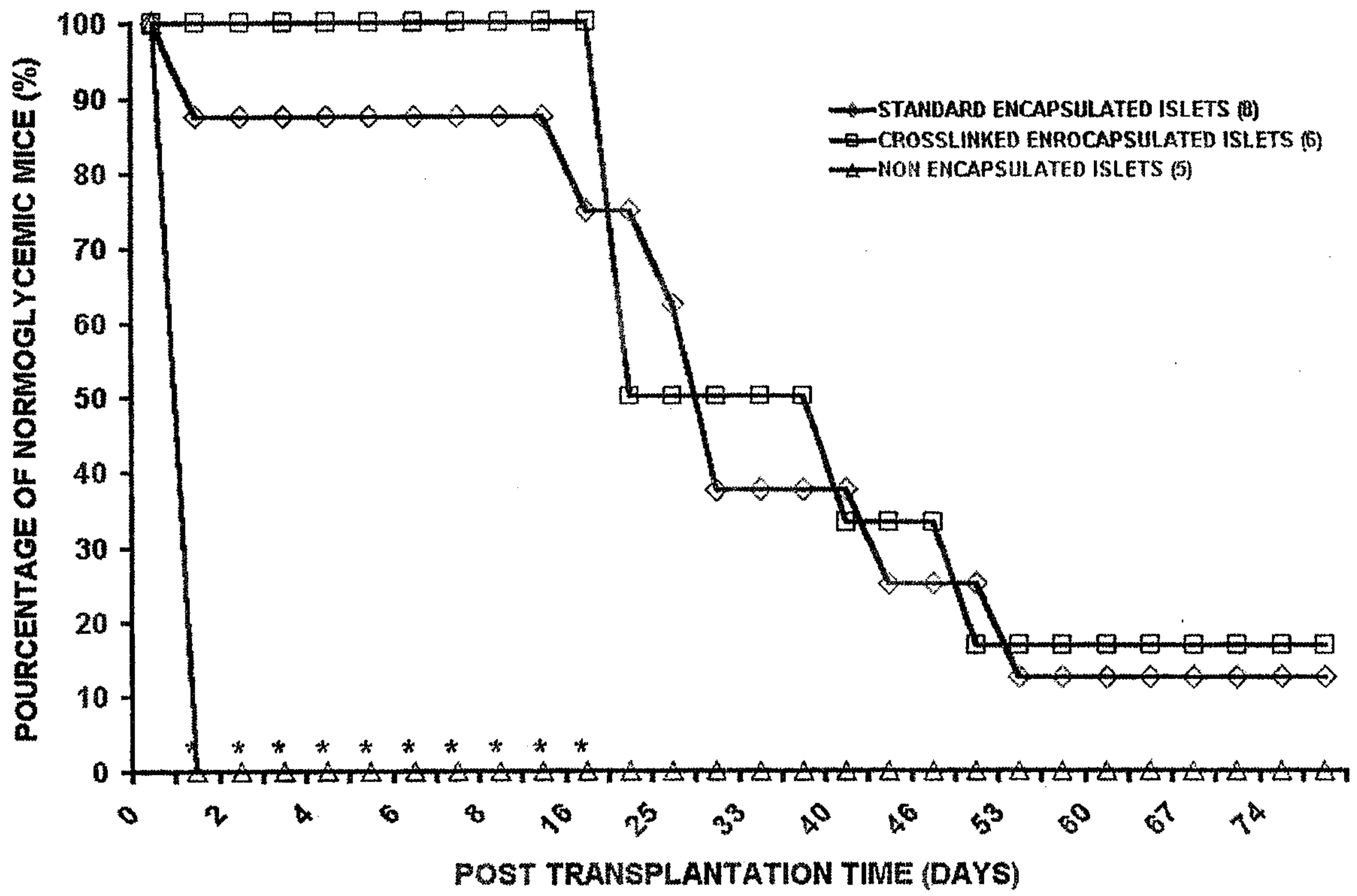

120
100
80
60
40
20
0
% VIABILITY
1
2
4
7
15
POST ENCAPSULATION TIME (DAYS)
*: p<0,001 vs 2 kJ/m2
γ: p<0,001 vs standards sans UVA
χ: p<0,05 vs 2 kJ/m2
δ: p<0,05 vs standards sans UVA


STANDARD ENCAPSULATED ISLETS
STANDARD ENCAPSULATED ISLETS / UVA
CROSSLINKED ENCAPSULATES ISLETS / UVA
% NECROTIC CENTER ISLET
100
80
60
40
20
0
1
2
4
7
POST ENCAPSULATION TIME (DAYS)

		Standard microcapsules			Crosslinked microcapsules	
Molecules	***Mw (Da)***	***R_η***	***K_{sec}***	***Profile***	***K_{sec}***	***Profile***
Dextran 2000000†	**$2X10^6$**	**34,2**	**0,0**	**excluded**	**0,0**	**excluded**
Dextran 19000	**19 000**	**3,4**	**0,0**	**excluded**	**0,1**	**excluded**
Dextran 4400‡	**4 400**	**1,7**	**1,0**	**included**	**1,0**	**included**
Bovine serum albumine	**66 000**	**3,4**	**0,0**	**excluded**	**0,1**	**excluded**
Ovalbumine	**45 000**	**2,9**	**0,0**	**excluded**	**0,1**	**excluded**
Carboxipeptidase	**35 250**	**2,7**	**0,9**	**included**	**1,0**	**included**

† Excluded molecule used as void volume

‡ Freely diffusing molecule used for the evaluation of total volume


100
90
80
70
60
50
40
30
20
10
0
POURCENTAGE OF NORMOGLYCEMIC MICE (%)
STANDARD ENCAPSULATED ISLETS (8)
CROSSLINKED ENROCAPSULATED ISLETS (6)
NON ENCAPSULATED ISLETS (5)
* * * * * * * * * *
0
2
4
6
8
16
25
33
40
46
53
60
67
74
POST TRANSPLANTATION TIME (DAYS)