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THE GROWTH OF POST-IMPLANTATION RAT EMBRYOS ON A STATIC CULTURE MEDIUM

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A dissertation submitted to the Faculty of Dentistry, University of the Witwatersrand, Johannesburg, in part-fulfilment of the requirements for the Degree of Master of Science in Dentistry

1982

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the Degree of Master of Science in Dentistry by course work in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Lauren Lasser

30th June 1982

Rosalind, Danielle and Dominic

Many thanks for the time I could
have spent with you

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ABSTRACT

Rat embryos of the post-implantation stage are grown on a static culture medium, for the purpose of assessing growth and development over a 2 day period. The technique used is referred to as the petri-dish culture chamber method. This involves the culture of rat embryos on rat serum, in a controlled environment. Post-implantation embryos from the 6,5 to 8 day stage, and consisting simply of the 3 germ layers, showed a 60% survival rate *in vitro* after 24 hours and 48 hours respectively. After a full 48 hours *in vitro* the embryos demonstrated well developed beating hearts, and a neural groove in the process of closing. The survival rate of embryos explanted at later stages was not as successful. The results compare favourably with the results of other workers, using the same method of culture. An advantage of the petri-dish culture chamber is that it allows for easy experimental access to the embryos at all times;a factor to be considered in any technique involving surgical intervention. Although more sophisticated methods have since superseded the static environment petri-dish technique, it is recommended here that this method should not be totally replaced by the more modern circulating culture medium technique.

INTRODUCTION

In 1888, Kastschenko claimed that mesenchyme of the head was derived from neural crest cells. This claim completely disrupted the neatly packaged triploblastic (or germ layer) theory, which was the recognised theory at that time. In 1947, De Beer working on Amblyostoma reported that neural crest cells gave rise to odontoblasts. Nearly one hundred years after the original study by Kastschenko and thirty five years after that of De Beer, there has to date been no conclusive information demonstrating unequivocal proof that the claim by De Beer is also true for mammals. Much experimental work has been performed on fish, reptiles and birds, but the difficulties associated with experimentation on mammalian embryos have until recently been regarded as insurmountable, because experimentation *in vivo* in mammals is not possible, and secondly, any experimentation *in vitro* must first overcome the problem of maintaining life without a placental blood supply. The advances in embryology, made by experimental procedures on chick embryos on the one hand (Noden 1973), together with the difficulties involved in experimentation on mammalian embryos on the other, created a climate in which it was acceptable to extrapolate from lower animals to man. However, the luxury of extrapolation became unacceptable, and the need for a mammalian experimental model was urgent. The first noteworthy breakthrough was the growth of rat embryos on a culture medium of heparinised rat plasma and embryo extract (Nicholas & Rudnick 1934). Following on this, a mammalian experimental model grown *in vitro*, and demonstrating growth and development closely comparable to embryos *in vivo*, finally became a reality (New 1967). Investigators (New 1966, 1967; New & Daniel 1969; Cockroft 1976; New & Coppola 1977) have reached this advanced stage working with rat embryos, although other animals were used.

It was for the purpose of making a mammalian experimental model available at the Oral and Dental Hospital, University of the Witwatersrand, that this project was undertaken. The foremost investigator in this field, D A T New (1966, 1967, 1970, 1978) has, in conjunction with a number of co-workers (New & Stein 1964; New & Daniel 1969; New & Coppola 1970, 1977) pioneered the techniques most widely used today. The aim of this project is to proceed along guidelines established by New. The technique uses a static culture medium in a petri-dish culture chamber.

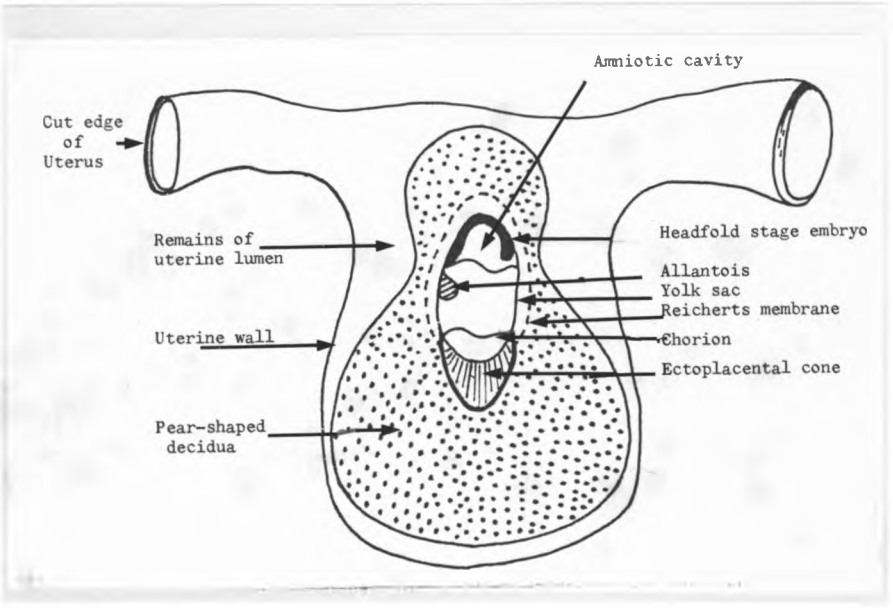
2. MATERIALS AND METHODS

The Wistar strain of rat is used in the present study. In order to obtain embryos at the desired stage, four female and one male rat, all of young breeding age, are placed together in a cage for a period of three nights. After this period, the females are removed and caged separately. Embryos are allowed to develop *in utero* to approximately the 7,5 day stage. The pregnant females are then sacrificed by cervical dislocation.

A mid-line abdominal incision is made exposing the abdominal viscera (figure 1). The bicornuate uterus of the rat is severed from its attachment to the vagina and is transferred to a dish of Tyrode saline solution (see 2.1.). The dish containing the gravid uterus is transferred to a sterile box.

The sterile box (figure 2) contains a thermostat to maintain the temperature at 37°C; a dissecting microscope; dissecting instruments; the petri-dish culture chambers; the Tyrode saline solution, as well as all the necessary glassware. The technique for sterilisation is described in section 2.4. Each nodular swelling is dissected free of the uterus and transferred to an operating dish containing blackened wax (see 2.2.). The operating dish containing the nodular swelling is placed on the dais of the dissecting microscope. All further dissection is carried out under the microscope.

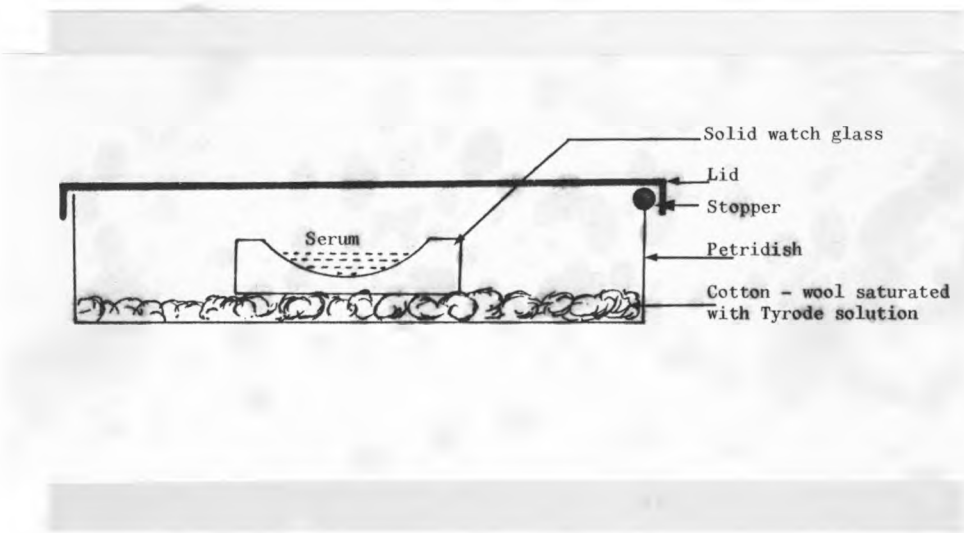
Removal of the uterine wall reveals the decidua, which is a pear-shaped mass of spongy tissue. The decidua is torn open to reveal the embryo covered by Reicherts membrane. The embryo adheres to one of the torn halves of the decidua (text figure 1).



Text figure 1 : Drawing of rat embryo *in situ*

The removal of Reicherts membrane is a delicate, yet necessary, procedure. The membrane is first slit with an eye knife and then stripped from the underlying yolk sac using watchmaker's forceps. The intact yolk sac containing the embryo is transferred to the culture serum in the petri-dish culture chamber.

The petri-dish culture chamber consists of a petri-dish and lid, containing a solid watch glass on a bed of cotton wool. The solid watch glass is used to house the serum culture medium, whilst the cotton wool, saturated with Tyrode solution maintains humidity within the dish. By using a stopper, the petri-dish lid is kept slightly raised to facilitate interchange of gases (text figure 2).



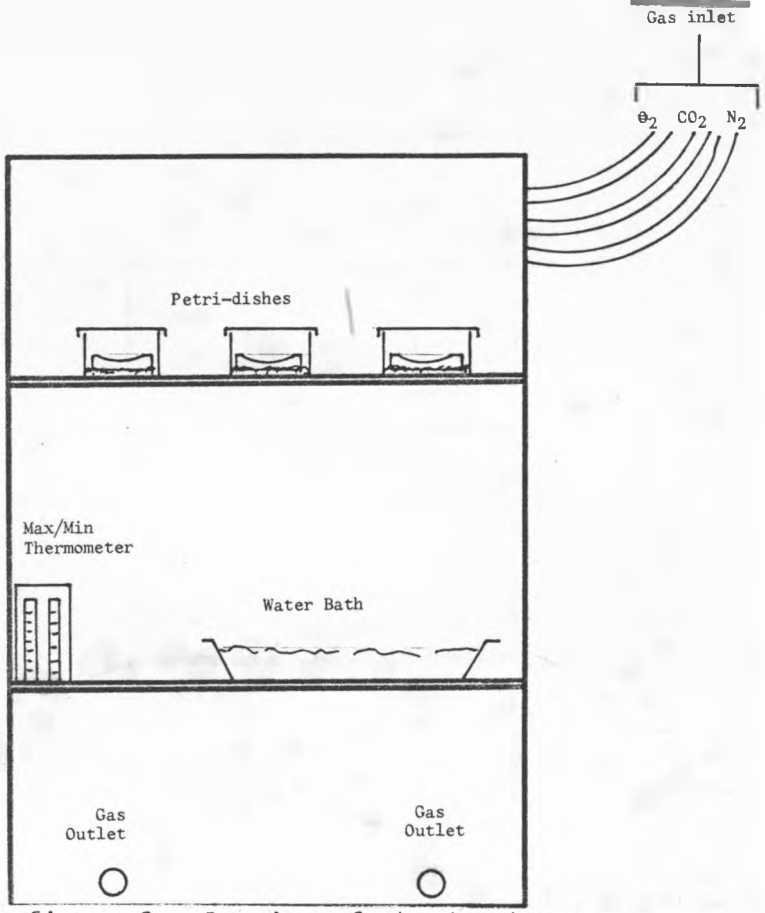
Text figure 2 : Drawing of the petri-dish culture chamber

The culture medium on which the embryos are grown is homologous rat serum containing 50μgms/ml Streptomycin (see 2.3.). The culture serum is prepared the day prior to the experiment and refrigerated at 4°C. The serum is transferred to the sterile box, where the addition of Streptomycin is carried out. Once the explanted embryo is transferred to the petri-dish chamber, the chamber is placed in the incubator.Embryos are left in the incubator for 24 hours or 48 hours respectively.

The incubator (text figure 3) contains a maximum and minimum thermometer, a water bath and three gas inlets. The temperature is maintained between 37°C and 39°C. The gas inlets are used for the introduction of oxygen, carbon dioxide and nitrogen, in the following recommended volumes :

Oxygen	20%	
Carbon Dioxide	5%	
Nitrogen;	75%	(New and Coppola 1970)

A gas flow meter is used to control the gas inflow.



Text figure 3 : Drawing of the Incubator

Each litter consists of between 10 and 14 embryos. On an average, 2 embryos are sacrificed immediately for examination of the external morphological features of the embryos and their associated membranes. The information thus obtained is used to establish an approximate age (table I). More accurate staging is only attempted after examination of the serial sections. Another 2 embryos are fixed in 10% formol saline in preparation for serial sectioning. These are referred to as the control embryos. Of the remaining embryos, approximately 2 per litter are rendered useless, either due to poor dissection or contamination. From an average sized litter, 6 embryos would be incubated. 3 embryos are removed from the incubator after 24 hours and 3 after 48 hours. All of these embryos are fixed in 10% formol saline and prepared for serial sectioning.

After fixing, the embryos are dehydrated in a graded series of ethyl alcohols; cleared in chloroform, and finally, paraffin-wax embedded. Serial sections are cut at 5μm and stained with haematoxylin and eosin.

The labelling of tissue sections was only done after careful examination of the serial sections of the embryos, of which the photographs are representative.

Table I

A tabulation of the criteria used for the external morphological examination of embryos for an estimation of age (compiled from information gained from Christie 1964, Witschii 1962, Long & Burlingame 1939).

No. of Somites	Age in Days	External Morphological Features
Presomite	7,5 - 8,5	Implantation complete Primary amniotic cyst and ectoplacental cone Primitive streak and head fold proliferation 3 germ layers are present Typical egg cylinder with amniotic and chorionic cavities not yet closed
1 - 6	9,0 - 10,0	Somites and neural groove are present The egg cylinder consists of 3 cavities: i Ectochoreonic cyst ii Exocoelom iii Amniotic Cavity Allantois is present Reverse curvature of embryo
7 - 10	10,0 - 10,5	Neural canal closed from level of 2nd to 6th somite Correction of curvature beginning Regular heart beat of 40 beats/min (Goss 1938) Otic pit forms
11 - 16	10,5 - 11,0	1st and 2nd branchial arches Correction of curvature complete Anterior neuropore closed Anterior limb bud just beginning
16 - 22	11,0 - 11,5	Posterior neuropore closed Otic pit closed Limb bud well developed Mandibular processes approaching midline but not fused

2.1. Tyrode Saline Solution

(Paul, 1970) is made up in the following manner :

Solution A

NaCl	2 gm
KCl	0,05 gm
CaCl	0,025 gm
$MgCl_2 6H_2O$	0,012 gm
$NaH_2PO_4H_2O$	0,012 gm

made up in 500 ml distilled water

Solution B

$NaHCO_3$	0,25 gm

in 480 ml distilled water

Solution C

Glucose	1 gm
Streptomycin	0,05 gm

made up in 20 ml distilled water

Solutions A and B are autoclaved at a temperature of 134°C and pressure of 2,16 kg/cm^2 for 15 minutes. Solution C is kept at 4°C. Immmediately prior to culturing, the three solutions are mixed together within the sterile box.

2.2. Blackened Wax

on which the dissection is performed is prepared as follows:

Wax is blackened with charcoal and oil soluble wax black, both of which are non-toxic substances.

2.3. Preparation of the Culture Medium

(New 1978) Blood is withdrawn from an homologous strain of rat. In this study a hypodermic needle was inserted directly into the heart chamber of an anaesthetised rat. An adult rat will produce about 10 ml of blood. This blood is then immediately centrifuged at 3 000 rpm for 15 minutes. The serum is decanted from the blood and heat inactivated at 56°C for 30 minutes. This process inactivates the complement, which is a

complex protein present in the plasma. This complement in conjunction with an antibody combines to cause dissolution of the bacterial membrane. Inactivation of the complement improves the capacity of the serum to support embryonic growth.

The final product of 'heat inactivated and immediately centrifuged'serum can be kept indefinitely if refrigerated. The serum used in the present study is prepared the day prior to the experiment and refrigerated at $4^{o}C$. 50μgm/ml of Streptomycin, made up in Tyrode saline solution,is added to the serum within the sterile box, immediately prior to the culturing.

2.4. Sterilisation

i The sterile box is swabbed down with 70% alcohol the day prior to the experiment. Sterility is maintained with the aid of an ultra violet light which is kept on overnight.

ii All the glassware in the box (figure 2) is sterilised by dry heat for 3 hours at $180^{o}C$. Prior to sterilisation the glassware is wrapped in brown paper and sealed. Unwrapping is done within the sterile box.

iii Sterilisation of solutions (see 2.1.)

3. RESULTS

Although numerous embryos were examined in order to demonstrate growth and development *in vitro*, the sections which appear in the text are all from embryos of the same litter, unless otherwise stated. The control embryos are examined immediately after explantation for staging on a morphological basis, and then fixed in preparation for serial sectioning. Growth and development of the embryos cultured for 24 hours and 48 hours respectively is compared with the developmental stage of the control embryo.

The control embryo in this series demonstrates the features of the very early post implantation period (figure 3) which extends from a 6,5 day old embryo to an 8 day embryo (Christie 1964, Witschii 1962). This particular embryo can be staged at about 7 days. The proliferation of cells in the headfold region supplies this stage with its name, i.e., headfold stage (figure 4). The section through amniotic cavity shows the two germ layers, i.e., ectoderm and endoderm, as well as the start of the mesodermal layer and the exocoelom. Owing to the small size of the embryo it is sectioned within the cylindrical yolk sac, the total length of egg cylinder being approximately 3 mm. If the egg cylinder had been sectioned along its length, a picture similar to the one depicted in figure 5 is demonstrated, the only difference being that at this early stage (7 days) the amnion and chorion are not fully developed, and the embryonic sac appears as a spindle-shaped sac constricted at its middle third.

After 24 hours in culture the viable embryos showed spherical yolk sacs (compare spindle shape *in vivo*) filled with clear fluid and a translucent embryo. The most prominent feature which has developed in the 24 hour culture period is the neural groove and its associated folds. The features of this neural groove are that it is open in its entirety (figure 6) and that it displays a reverse curvature which is typical for Rodents at this stage of development (figure 7).

Until the 9 day stage the dorsal neural groove is concave in curvature (unlike human embryos which are dorsally convex from the start) and the ventrally situated heart lies on the convex curvature (figure 14, which is taken from a different litter to the one described, but because of the longitudinal view, demonstrates the point more clearly).

The section (figure 7) cut through the tail and headfold of an embryo, shows that the neural groove of the tail faces the neural groove of the head, thus demonstrating the dorsally concave curvature. Another feature evident within these sections is the convex curvature of the 'V' shaped cranial neural folds - while the caudal neural folds demonstrate a more cup shaped groove (figure 7). All these features described compare very favourably with *in vivo* embryos at the same stage. Associated with the neural groove are the somites. Microscopically they show up as clusters of cells, radiating peripherally in a starlike fashion (figure 6).

The second most obvious development at this stage is that of the heart. On examining the embryo morphologically the heart is seen to pulsate rather than throb. Microscopic examination reveals the 'S' bend of the tubular heart (figure 8).

The third featural change is the presence of primitive fore-gut and the start of branchial pouch formation (figure 8). (Figure 14, taken from a different litter, demonstrates the formation of branchial groove. This embryo was cultured *in vitro* for 48 hours starting at approximately the 6,5 day stage).

The final stage at which the embryos are examined is after 48 hours in culture. The most successful embryos at this stage (judged according to degree of growth and development) showed spontaneous adhesion of the ectoplacental cone to the glass surface of the watch glass, whilst the developing embryo within its membranes floats just below the surface of the serum culture medium.

A section through the cranial region of an embryo which was grown *in vitro* for a period of 48 hours reveals that the neural groove has

closed to form the neural tube (figure 9). The neural tube is closed from the level of the second to the sixth somite, a feature which places the embryo at an age of 9,5 to 10 days. Further correction of the reversed curvature pattern, unique to the Rodent, is seen. This is difficult to demonstrate in a histological section though in some sections a closed neural tube and an open neural groove in the same section are taken as an indication that the body of the embryo is bending to one side in relation to the cranial region (figure 10).

The heart at this stage is well developed and the mandibular process is developing lateral to the heart. The development of a good circulation is demonstrated by the growth of allantois towards the ectoplacental cone to form the chorioallantois (figure 11). The embryos grown in culture for 48 hours show a comparable rate of growth to similar stage embryos *in vivo* In this study, post-implantation embryos of the presomite stage and/or those of up to 3 somites, show the best (60%) survival rate. Embryos explanted after the 8 day stage did not demonstrate a similar success rate with this method. This is almost certainly due to the fact that, in the course of this project, the methodology was not altered to suit the growth requirements of the older embryo.

In the course of this study, certain changes which were made enhanced the survival rate of the embryos in culture. For example, better results were obtained when the petri dish lid was raised (see Materials and Methods). Secondly, results were improved by creating an even temperature for the embryo during its transfer (a) from the *in vivo* situation to the Tyrode solution (b) to serum culture (c) to incubator. Thirdly, maintaining the humidity was essential for the survival of the embryos.

An analysis of the results (Table II) only takes into account those results which were achieved after making the above adjustments.

Table II

The percentage survival of different stages of embryos over a predetermined period

No. of embryos incubated	Somite stage at start of culture	Somite stage at termination of culture after 24 hrs	48 hrs	% survival in culture after 24 hrs	48 hrs	Increase in number of somites after 24 hrs	48 hrs
18	0 - 2	6 - 8	10 - 15	90%	60%	6	10 - 13
12	9 - 20	15-26		30%		6	
6	20 - 30	26-33		25%		3-6	

In assessing survival, a number of factors are taken into consideration. On morphological examination, the tissues should present a translucent texture, and the yolk sac should be filled with a clear fluid; a heart beat must be present in a 9 day embryo. On histological examination there should be no degeneration and no abnormal developments in organogenesis. Embryos should demonstrate a growth rate comparable to embryos of the same age grown *in vivo*.

4. DISCUSSION

Embryo cultures have been attempted with guinea pig, mouse, rabbit and rat. Of these, cultures of rat embryos demonstrate the best results. New (1966) used both the Hooded and Wistar strain of rat, with equal success. The Wistar strain was chosen for this study because of its availability at this institution. It breeds easily in the experimental situation and displays qualities of hardiness and ease of handling. Cervical dislocation is the method used for sacrificing the pregnant females. Other methods, using gas or drugs, can adversely affect the embryos.

In the present study, the appearance of a vaginal plug is not used as a means of deciding whether the rats had mated. Instead, males and females were caged together for a full 3 nights before being separated. Signs of pregnancy are sought for during the ensuing 7 day period. The reason for this deviation from New's method is because it was often difficult to determine the presence of the plug, and secondly, the mere presence of the plug did not ensure that fertilisation had taken place.

The staging of embryonic development in this study is along guidelines established by Long & Burlingame (1939), Witschii (1962) and Christie (1964). All these workers use the appearance of a vaginal plug as a starting point for ageing embryos. At 12h00 of the morning after a vaginal plug appears, the embryo is standardised at 12 hours of age. Using this method could therefore create an error of approximately 8 hours growth. Furthermore, it has been reported that embryos of the same litter can vary from 1 to 6 somites in growth (Long & Burlingame 1939). As a 12 hour period *in vitro* can correspond to the growth of from 8 to 10 somites, the resultant error can be considerable. Juurlink & Federoff (1979), working with mouse embryos, regard the appearance of a post-copulatory plug as denoting the start of day '0' of gestation.

The uncertainty surrounding the ageing of embryos (in days) is the main reason why, in this study, age in days is not emphasised, although an attempt has been made to estimate age along parameters set out by Long & Burlingame (1939), Witschii (1962) and Christie (1961).

The second deviation from New's technique is in the making up of the Streptomycin in the solution of Tyrode saline. In effect, this altered two aspects of the experiment. Firstly, the working solution contains Streptomycin, so that the transfer and dissection of the embryos takes place in an antibacterial environment. Secondly, the addition of Streptomycin to the serum, means that Tyrode saline is also added to the culture serum. It is felt that the minute amount of Tyrode saline introduced is negligible and has no effect on the embryo. This corresponds to a report on the use of different culture media (New 1978), that additives to the culture serum of this nature made little or no difference to the result.

A critical factor affecting *in vitro* development of embryos, is the gas interchange from atmosphere to serum to embryo (New & Coppola 1970). Poor gaseous diffusion is probably the chief cause of failure in systems using a static culture medium. It is found that, on placing the petri-dish chamber in the incubator, moisture, which collects on the lid of the dish, trickles to the surrounding rim, creating a further gas barrier within a very short space of time. In order to eliminate this, a stopper is placed beneath the lid, thus allowing the better interchange of gases. This adjustment however, does increase the risk of drying out within the culture chamber. Regular monitoring is necessary to ensure that drying out does not occur.

The results of the present series show that the very young post-implantation embryos (post 6,5 days) have the best survival rate (60%) on a static culture medium (Table 1). New (1966) also reports a 60% survival rate for embryos at the same stage on a static culture media. However, it is not made clear whether

New's figure is 60% of all embryos excised, or 60% of those incubated. Although the younger embryos show a better survival rate using this technique, it is more difficult to dissect them free of their membranes without causing damage to the underlying yolk sac and embryo; with the result that a greater number of the embryos are spoiled during dissection.

An observation of this study, is that embryos which survived their respective periods *in vivo* (i.e. 24 hours and 48 hours respectively) demonstrated the potential for further growth and development; the criteria for this assessment being the presence of a regular heartbeat with a good circulation (New 1978). Embryos explanted at stages beyond 8 days showed a relatively poor survival rate. The reason for this is probably because the gas concentrations were not adjusted to suit the requirements of the older stage embryos. A further study could rectify this condition.

The finding, that embryos *in vitro* demonstrate spherical yolk sacs, as compared to spindle-shaped yolk sacs of the *in vivo* specimen, may be due to two reasons. The first of these, is the theory proposed by New & Daniel (1969), that the *in vivo* embryo is subjected to a tissue pressure which plays a part in the determination of shape. The second reason, and the one that is formulated as a result of this study, is the absence *in vitro* of Reicherts membrane. Reicherts membrane, *in vivo* , is a tough pink membrane, which is present only in the Rodent (Snell 1956). It surrounds the embryo in its yolk sac, and is continuous with the trophoblastic layer of the ectoplacental cone. The inability of Reicherts membrane to grow *in vitro* restricts embryonic growth (New 1966), and it is therefore removed before culturing.

The phenomenon of spontaneous adhesion referred to in the results of the present study has not been reported on elsewhere in the literature. This phenomenon when it occurs, is associated with a good growth and development, including a correction of curvature which is normal for this stage of embryo (9 - 16 somites). It is suggested in this study, that the adhesion supplies an anchorage

which is beneficial to the process of curvature correction. This corresponds to the observation by New (1978) that embryos cultured in circulating media - in which they are anchored by Reicherts membrane to a strip of gauze - demonstrate complete correction of curvature(Deuchar 1971)

The two most encouraging aspects of this study are firstly, the finding that a static culture medium can support the growth of good quality tissue, and secondly, that the growth rate achieved *in vitro* closely resembles that of the embryo *in vivo* (according to staging criteria of Long & Burlingame 1939, Witschii 1962, Christie 1964). Though the 60% survival rate appears at first to be rather low for a 48 hour culture period, the factors used to assess survival were rather stringent. The initial appearance of the embryo and its membranes should demonstrate a translucent texture, and the yolk sac should be filled with a clear fluid - the absence of these two findings is often associated with degeneration. A heartbeat should be visible on external morphological examination - though the presence of a heartbeat alone does not preclude survival. The contractile nature of heart muscle could cause a heartbeat to persist in an embryo which is otherwise non-viable. On histological examination no(or very minimal) degeneration should be present. The embryo should demonstrate a growth rate comparable to *in vivo* embryos of the same age, and an organogenesis which is totally normal in character. Any embryo which did not demonstrate at least four out of the five criteria mentioned above, was regarded as a non-surviving embryo. Embryos which showed abnormal development (even in the presence of a heartbeat and good tissue quality) were discarded as non-survivors. Abnormal developments were more noticeable in embryos which were explanted after the 9 day stage.

The poor survival rate of embryos explanted after the 9 somite stage, is simply due to the fact that the study was not geared towards the older embryos. However, Table II is presented as an analysis of the entire project for the purpose of completeness. The only deduction that can be made from a comparison of the various ages of embryos, is that the embryos explanted at an older age

do not fare as well *in vitro* under the same environmental conditions as the younger embryos. The most important environmental adjustment necessary is in the percentage gas volume, and for static culture medium, in the gas pressure as well.

New (1978) states that a complete assessment of embryonic growth and development can only be carried out in conjunction with crown-rump measurements and protein assays of pre- and post-cultured embryos. Because the embryos dealt with in this study are 10 days old or younger, with a crown-rump measurement not exceeding 3 mm., crown-rump measurements were found to be impractical. Also, in the process of removing the membranes, and then drying the embryo, a tremendous amount of distortion occurred. The correction of the curvature, which is an ongoing process occurring from the 9th to the 16th somite stage, made the measurements inaccurate. Finally, because of the absence of tissue pressure *in vitro* and because of the removal of Reicherts membrane for *in vitro* culture, the yolk sac, and consequently the embryo, demonstrate a change in shape. Therefore, it is felt that crown-rump measurements *in vitro* are not comparable with those *in vivo*. Protein assays are a very accurate means of determining protein synthesis and thus growth, but the difficulty of separating the young embryos from their membranes remains to be overcome. Another problem associated with protein assays, is that it can never be ascertained whether or not the protein measured is derived from abnormal organogenesis.

The petri-dish culture chamber is an uncomplicated technique, with a number of advantages. However it has since been surpassed. Using more complex equipment, the culture medium is made to circulate (New 1967, 1978, Robkin & Shepard 1972) and gases are introduced directly into the culture serum in carefully controlled proportions (New & Coppola 1970, New, Coppola & Cockroft 1976). Under these conditions, the survival rate in culture is increased to 90%. The period for which embryos are kept viable and developing normally is increased to 4 - 5 days, and includes all stages of organogenesis from egg cylinder to the 40 somite stage (New 1978). The main advantages of the technique employing a circulating medium, is that the developing embryo can be constantly monitored, to the extent that films have been made of the embryo in culture. It

is also an ideal situation for testing the effects of various agents on embryonic development.

In conclusion, acknowledgement is made to the better efficiency of the circulating medium and to the fact that embryos cultured therein grow and develop for longer periods *in vitro*. Nonetheless, the static culture medium can uphold its use amongst modern investigators. Firstly, it makes use of uncomplicated and non-costly equipment and secondly, the petri-dish culture chamber allows easy access to the embryo at all times during the culture period. For this reason, it would be particularly useful to any experimental technique involving a surgical procedure; for example, in experiments involving transplantation of neural crest cells from one area of the embryo to another, or the surgical introduction to an embryo of radioactively treated neural crest cells, in order to determine their path of migration and possible destination into dental pulp.

5. CONCLUSION

The use of the petri-dish culture chamber has made it possible to fulfil the aim of this project, which was to make available to the Oral and Dental Hospital at the University of the Witwatersrand, a mammalian experimental animal Although a maximum *in vitro* growth period of 48 hours is used, the embryos showed the potential for continued growth and development. It is hoped that this finding will stimulate a further study, using increased periods of growth on culture. The completion of the task opens the way for a future investigation into the claim by De Beer in 1947, that odontoblasts are of neural crest origin. The amount of organogenesis which is taking place over short periods of time, together with the finding that embryos can grow and develop *in vitro* at comparable rates to that of *in vivo* embryos, makes it possible to use this system of culturing for animal experimentation. Three factors in favour of the petri-dish culture chamber are firstly, that it is inexpensive and involves the use of uncomplicated equipment; secondly, that the growth and development is of a good quality and thirdly, that the system allows for easy access to the embryos at all times during culturing - a condition which does not prevail in the circulating culture medium technique. It is predicted that this last factor will ensure the continued use of the petri-dish culture chamber for mammalian animal experimentation, particularly where direct surgical intervention is contemplated.

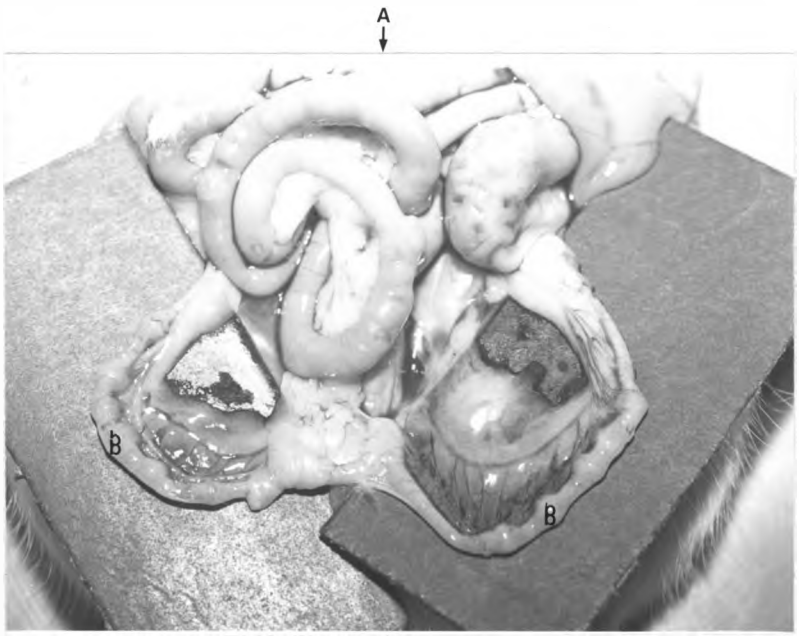


Figure 1 : A pregnant rat showing the midline abdominal incision. Abdominal incision A. Bicornuate uterus B.



Figure 2 : Sterile box which is used in this experiment.

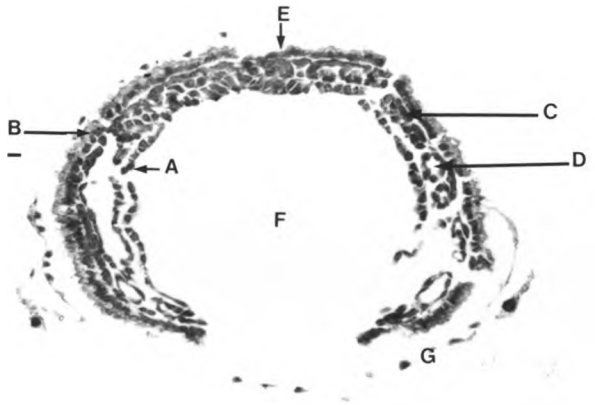


Figure 3 : Cross-section through amniotic cavity of 7 day old embryo Mag x 12,50
showing Ectoderm A. Endoderm B. Mesoderm C. Exocoelom D.
Primitive streak region E. Amniotic cavity F.
Cut edge of yolk sac G.

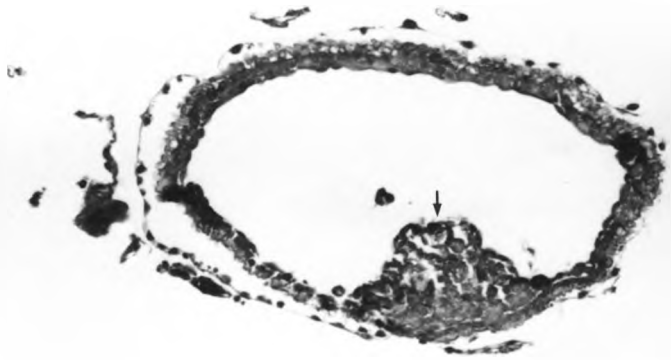


Figure 4 : Cross-section through amniotic cavity of 7 day old embryo Mag x 12,50
showing head fold (arrow)

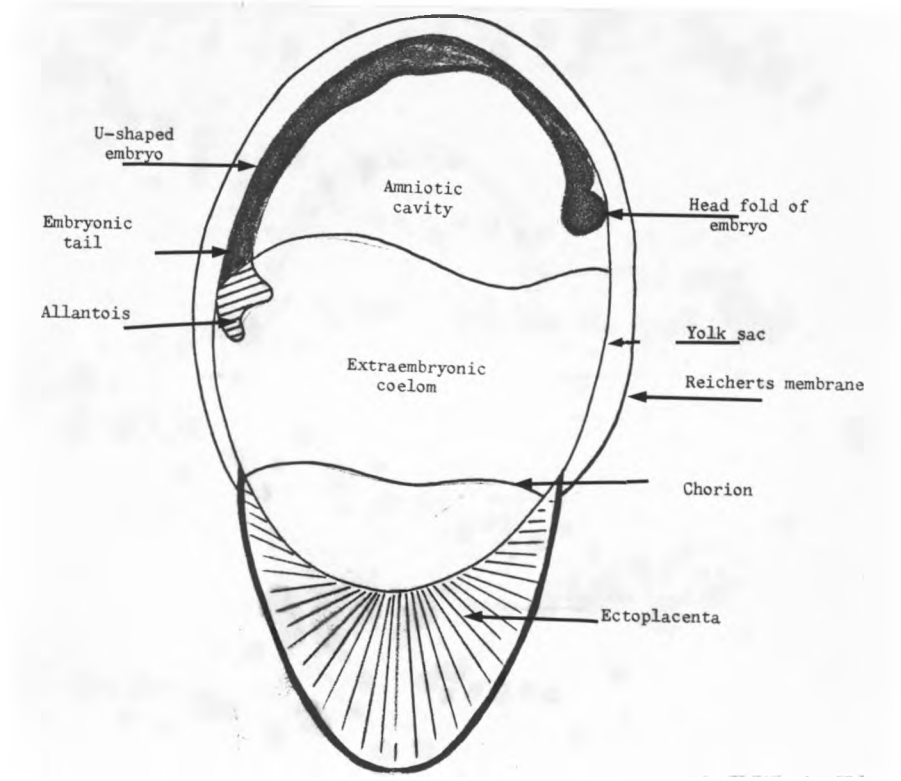


Figure 5 : Longitudinal drawing of the egg cylinder showing the embryo and its associated membranes



Figure 6 : Longitudinal section through the middle one-third of an embryo which was grown *in vitro* for 24 hours showing open neural groove A. Mag x 10
Somite development B.

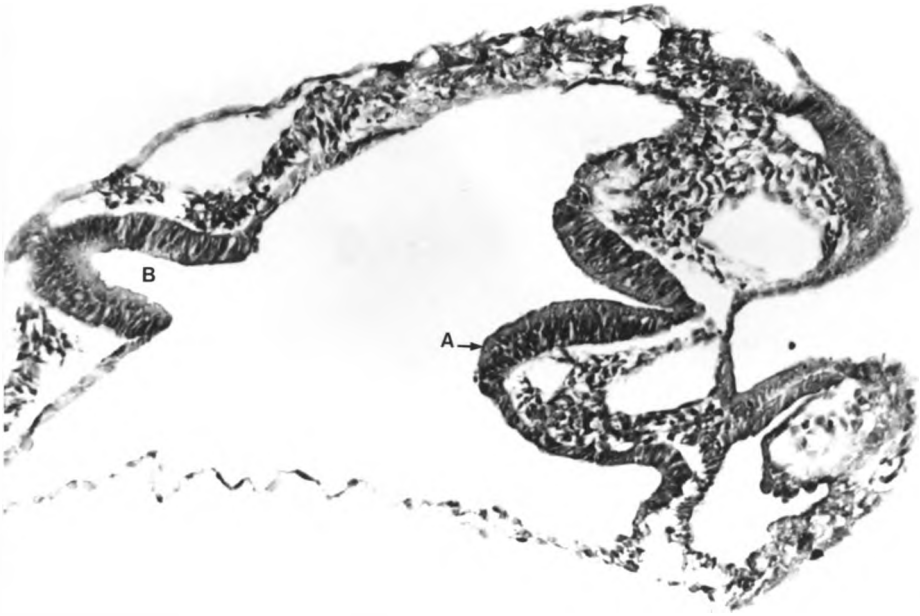


Figure 7 : Cross-section through an embryo cultured for 24 hours *in vitro* Mag x10,00
showing Cranial neural groove and its convex neural fold (arrow) A. caudal, cup-shaped neural groove B.



Figure 8 : Cross-section of an embryo after 24 hours in culture Mag x10,00
showing Tubular heart at 'S' bend A. Foregut B. Allantoic development outside of amniotic cavity C.



Figure 9 : Cross-section of embryo cultured *in vitro* for 48 hours Mag x 5,00
showing Closed neural tube A.



Figure 10 : Cross-section of embryo cultured *in vitro* for 48 hours Mag x 5,00
showing Closed neural tube A. Open neural groove B.

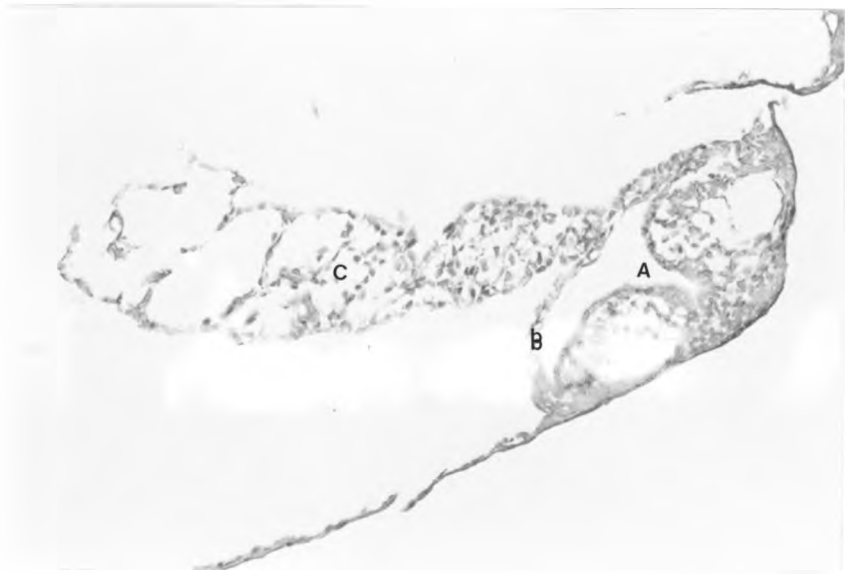


Figure 11 : Cross-section through tail of embryo grown *in vitro* for 48 hours Mag x 0,00 showing Tail neural groove A. Cut edge of amnion B. Allantoic blood capillaries C.

The figures which follow are taken from a different litter to the one previously described, but serve to emphasise a few more features of the developing embryo. (They are to be referred to as the second series of figures).

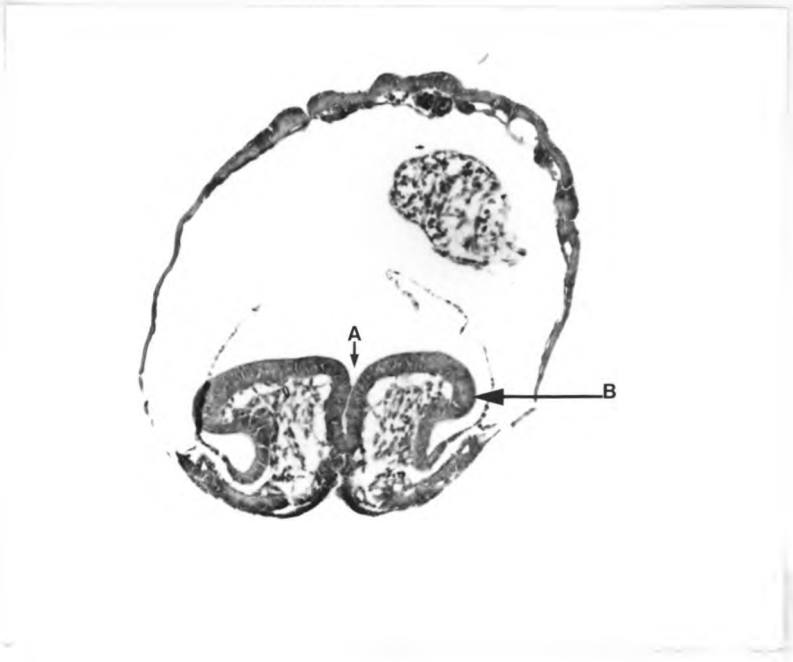


Figure 12 : Cross-section of an embryo of the second series after 24 hours in culture. Age 8,0-8,5 days. Mag x 5,00 showing Neural groove A. Neural folds B.

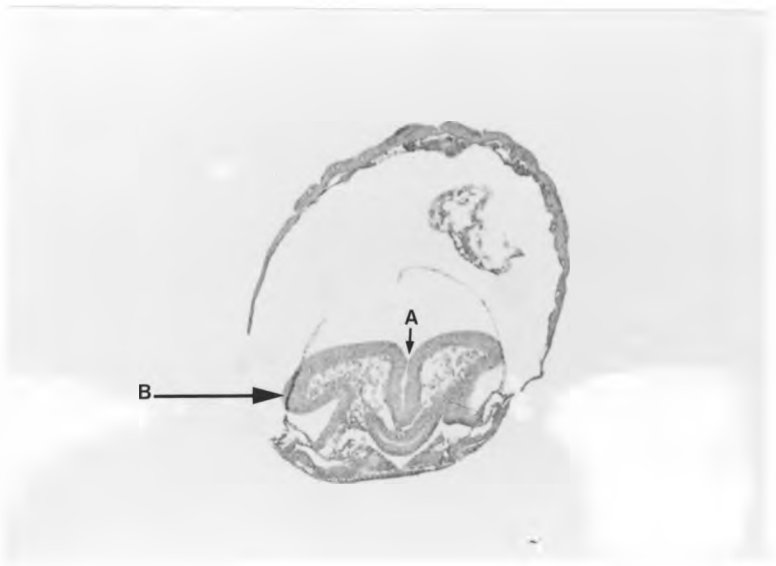


Figure 13 : Cross-section of an embryo of the second series after 24 hours in culture. Age 8,0-8,5 days Mag x 5,00 showing Neural groove A. Neural folds B.

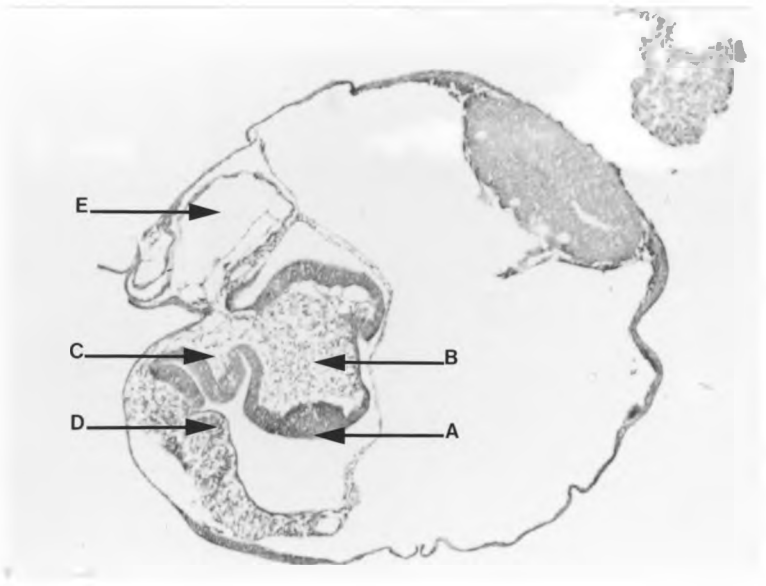


Figure 14 : Longitudinal section of an embryo of the second series grown in culture for 48 hours Mag x 5,00 Age 9,0 to 9,5 days. Showing Neural Fold A. Telencephalon B. 1st Branchial arch C. Somites D. Ventrally situated heart vesicle E.

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