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Thyroid Culture from Monolayer to Closed Follicles

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1. Introduction

1.1. Morphology

The thyroid is an endocrine gland formed by two lobes (Figure 1A L) located on each side of the trachea and the larynx; they are joined by an isthmus (ultimo-branchial origin) (Figure 1A clear arrow) located between the trachea's second and third cartilage rings (Figure 1A black arrow).

1.1.1. Histology

A fibrous connective tissue capsule covers each lobe from where the septa go inside, partially dividing the glandular parenchyma containing a very developed network of capillaries surrounding the follicles and irrigating the glandular parenchyma. The gland also contains adipocytes, nerve fibres, mastocytes and occasionally lymphocytes and macrophages [2].

The thyroid parenchyma mainly consists of follicles which are the thyroid's functional unit (Figure 1B). It has an oval or spherical structure whose wall consists of a layer of cubic epithelial cells (thyrocytes) (Figure 1B black arrow) surrounding a viscous solution of proteins called colloid (Figure 1B Co) [2,3] containing 80% thyroglobulin (Tg) or thyroid hormone [4,5]. Follicle size varies according to an individual's age, its localisation in the gland and animal species; for example, diameter varies from 50 to 150 μm in rats and mice where peripheral follicles are larger than the central ones (Figure 1A L), whilst diameter varies from 150 to 500 μm in humans and pigs, the largest ones occurring towards the inside of the gland, even though their location could vary [6,2]. As well as follicle cells or thyrocytes, it has been found that 1% to 2% of neural crest cells in different mammals' thyroids are parafollicular or clear cells, appearing clearer in different types of histological staining. These are located at the base of follicles but do not come into contact with the colloid and secrete calcitonin [7].
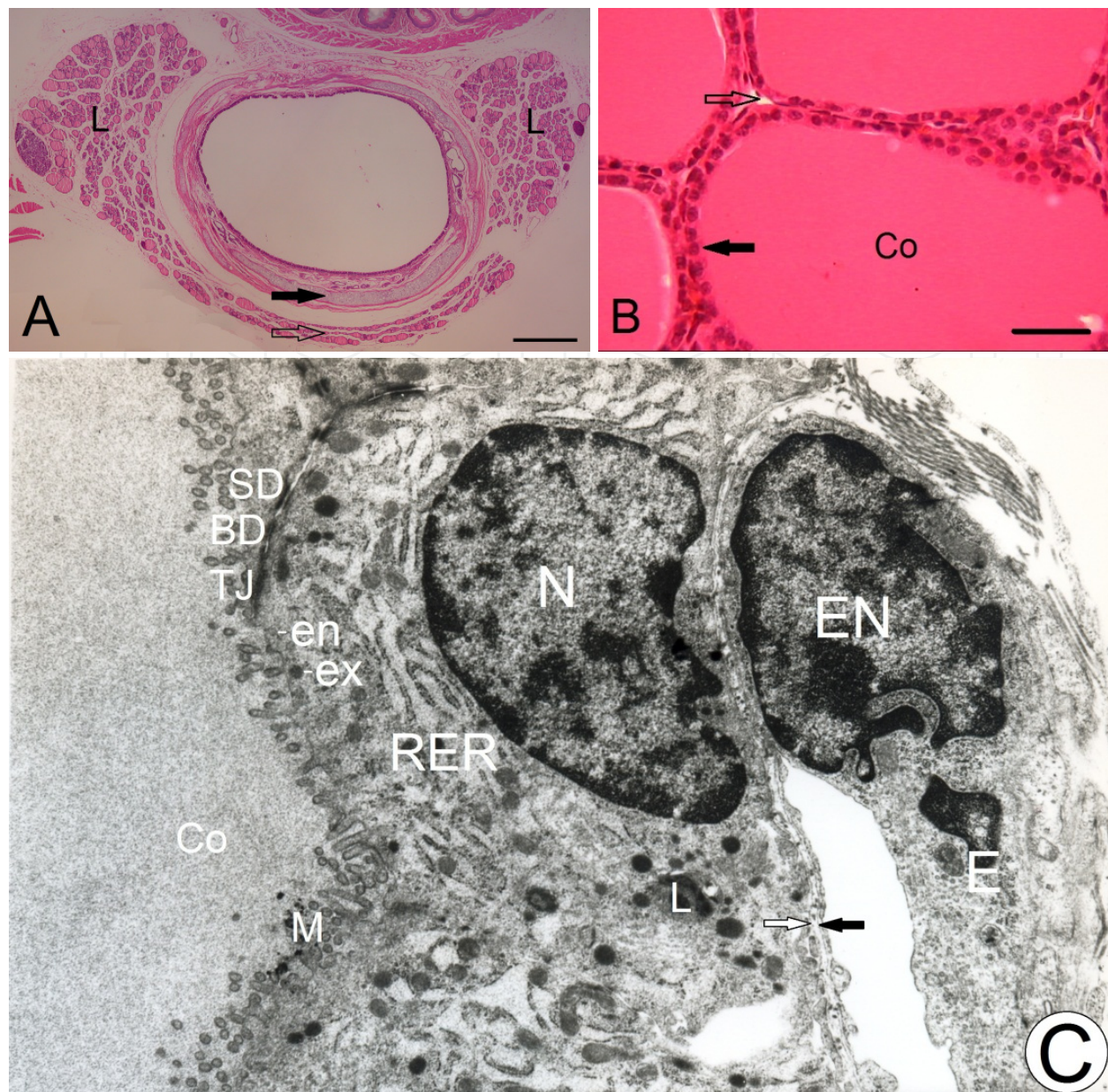
Figure 1. A. Histological cross-section of mouse trachea, showing cartilage (black arrow) and mouse thyroid lobes (L) and isthmus (clear arrow) adhering to connective tissue between cartilage and thyroid. The thyroid parenchyma consists of follicles which look like spherical or oval structures. **B.** The appearance of pig follicles in the lobe's central region. Each follicle consists of simple cubic epithelium (black arrow) limits the follicle centre full of colloid (Co) which follicular cells or thyrocytes secrete. The capillaries surrounding the follicles can be seen (clear arrow). **C.** Rat thyrocyte ultra-structure or cytology. The rugose endoplasmic reticulum (RER) can be seen around the nucleus (N) and Golgi complex in supranuclear position; these organelles and lysosomes (L) occupy the cells' base region. Different vesicles can be seen at apical level, from exocytosis (ex) being denser than electrons and endocytosis (en) and having the same density as electrons and colloid (Co), and the apical membrane forming microvellosities (M) in contact with colloid in the centre of the follicles. The binding complex can be observed in the lateral membrane at apical level: tight junctions (TJ) followed by belt desomosome (BD) and spot desmosome (SD). The thyrocytes' basement membrane or basal lamina (white arrow) is in close contact with the endothelium's (E) fenestrated capillaries' basement membrane (black arrow); the endothelium cell nucleus (EN) can be seen (A and B H-E, MO. Scale bar A 1mm, B 40 μm. C TEM. 7300X).

1.1.2. Ultra-structure

Endocrine gland cells are usually not polarised with their central nucleus and organelles; however, thyrocytes are exceptionally polarised endocrine cells and the nucleus and organelles are located in the cell's basal region like exocrine epithelium cells. This is due to the larynx's embryonic development by polarised cells involuting inside it. Such morphological and exocrine functional characteristics are conserved within follicles for thyroid hormone synthesis, storing and secretion (Figure 1C). The thyrocytes' basal membrane is directly related to the follicles' basement membrane or basal lamina (Figure 1C white arrow), in turn, being in direct contact with the fenestrated capillaries' endothelial (Figure 1C E) basement membrane (Figure 1C black arrow). The thyrocytes' apical membrane is in direct contact with colloid forming microvellosities (Figure 1C M) whose length and amount vary according to a gland's functional state [8]. Exocytotic vesicles (Figure 1C ex) can be seen in the apical region, some of them being more electron dense than less electron dense endocytic vesicles (Figure 1C en) and some coated at the base of microvellosities [5].

The thyrocytes' lateral membranes have binding complexes in the apical region formed by tight junction, belt desmosome and spot desmosome (Figure 1C TJ, BD, SD) isolating and separating colloid from the intercellular spaces and basolateral apical membranes [9].

The thyrocytes' nucleus is surrounded by abundant RER in the cells' basal region (Figure 1C RER) and the GC is in the supra-nuclear region in normal physiological conditions (Figure 1C N). The lysosomes are located in the thyrocytes' basal media region (Figure 1C L). The mitochondria are distributed throughout the whole cell.

The thyrocytes form depends on their functional state; they are cubic in normal conditions (euthyroid morphology) and have the aforementioned ultra-structure (Figure 1C). Without thyrotrophic or thyrotropin-stimulating hormone (TSH), endocytic vesicles disappear at the beginning and exocytosis increases and microvellosities become reduced. The lysosomes increase in size and have very heterogeneous content following several days without TSH; the follicular cavity increases after a few days and cells become thin and atrophied because the RER and GC become reduced. Such cells disappear following 20 or 30 days' suppression of TSH (hypothyroid morphology). A rapid increase in exocytic vesicles (micropendocytosis) occurs when the gland is stimulated by TSH and the apical membrane forms cytoplasmic expansions or pseudopods forming large macroendocytic vesicles called colloid droplets; such vesicles merge with lysosomes which migrate to the cells' apical region [10]. When TSH stimulation is sustained for more than 5 days, microvellosities' length and amount increase, follicular cavities become reduced and the thyrocytes become cylindrical and hypertrophied because the RER and GC increase, occupying almost the whole of a cell (hyperthyroid morphology) [2,8,10,11].

1.2. Function

T_3 (3,5,3'-triiodine-thyronine) and T_4 thyroid hormone (3,5,3',5'- tetraiodine-thyronine) synthesis reflects thyrocytes' follicular morphology and ultra-structure which can be divided into 3 stages (Figure 2 circles): stage 1, Tg synthesis and colloid secretion at
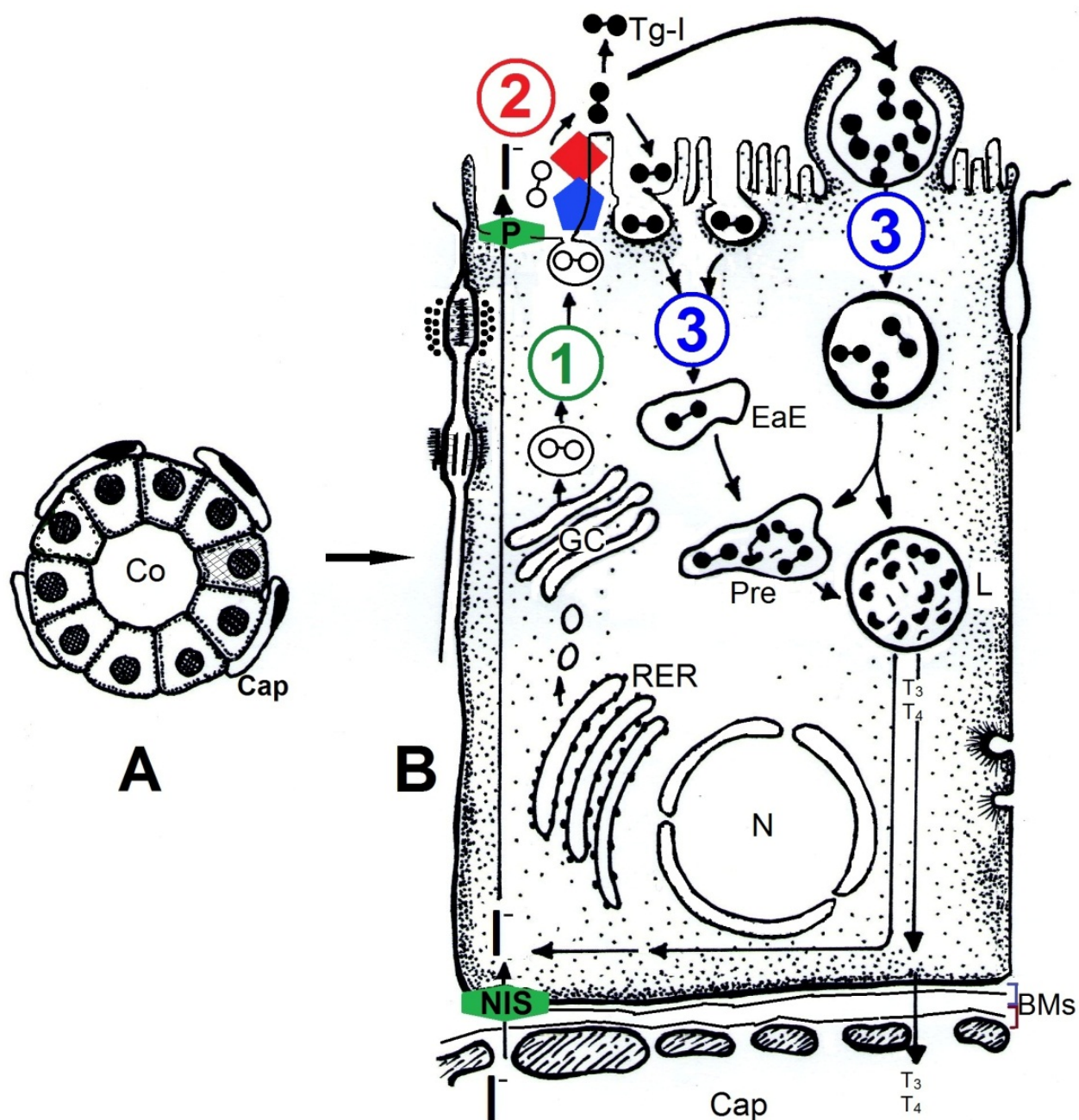
Figure 2. A. A diagram of a thyroid follicle surrounded by capillaries (Cap); Co: colloid. **B.** A diagram of a thyrocyte showing the physiology of thyroid hormone synthesis. Iodide is captured in the basement membrane by the *sodium/*iodide symporter or Na^+/I^- symporter (NIS) and rapidly transported to colloid, mainly via pendrin (P); it is used there for thyroid hormone synthesis. Tg is synthsised in the RER and *N*-osidic glycosylation culminates in the GC; Tg is secreted to colloid by exocytic vesicles (green circle 1). Once in colloid, thyroperoxidase (blue) with thyroid oxidase 1 or 2 (red) fixes iodide to Tg (red circle 2) forming iodine-thyronine on Tg (Tg-I). When thyroid hormones are required, Tg-I is endocyted by microvesicles or macrovesicles (blue circle 3) when TSH stimulates the thyroid. Tg-I becomes degraded by lysosomal enzymes (L) releasing T_3 and T_4 into the blood stream. N: nucleus; RER: rugose endoplasmic reticle; GC: Golgi complex; I^-: ion iodide; NIS: Na^+/I^- symporter; EeE: early endosome; Pre: prelysosome or late endosome; L: lysosome; BMs: basements membranes or basals laminas; T_3 and T_4: thyroid hormones. Diagram modified from Spinel (2003) [12].

intracellular level; stage 2, iodide accumulation and its organification regarding Tg in colloid at thyrocyte extracellular level; and stage 3, endocytosis and intracellular hormone secretion [5,12].

The first stage occurs at intracellular level, Tg is synthesised and glycosylated (*N*-osidic glycosylation) in RER and then glycosylation in culminates in the GC. Tg is transported in exocytic vesicles which emerge from the GC (Figure 2 green circle 1) and is released to the colloid [5]. Iodide accumulation takes place in the basal membrane via the sodium/iodide symporter (NIS) or Na^+/I^- symporter (Figure 2 NIS) [13]. It then passes through colloid via pendrin (I^-/Cl^- apical exchanger) [14] (Figure 2 P), regulated by the apical region's ClC-5 channel [15].

The second stage happens at extracellular level. Thyroperoxidase (TPO) is found in the base of microvellosities anchored to the apical membrane which oxides iodide (Figure 2 blue) and thyroid oxidases 1 or 2 (Duox1 and 2) forming H_2O_2 (Figure 2 red). TPO fixes one or two iodines on specific Tg thyrosins in colloid (iodide organification), thereby forming mono- and di-iodinethyrosins (Figure 2 circle 2). TPO couples the iodinethyrosins, producing iodine-thyronine or T_3 and T_4 hormones on Tg19S or thyroid prohormone (Figure 2 Tg-I); in both processes TPO reduces H_2O_2 [16,17].

The third stage is intracellular. Tg19S is endocyted [10] (Figure 2 blue circle 3) and degraded in prelysosomes [18] and in lysosomes (Figure2 Pre, L), releasing the hormones which become diffused through the basal membrane to the blood stream [19] where they are transported by three families of blood proteins to an organism's cells [20,21].

Due to the thyroid's morphological characteristics and its function, "it is an exquisitely regulated gland", [22]. Its function is essentially controlled by the hypothalamus-hypophysis and also by the nervous system and other thyroid systems [22]. Thyroid gland function and growth is controlled by TSH secreted by adenohypophysis thyreotropic cells. TSH secretion is stimulated by thyrotropin releasing hormones (TRH) secreted by the hypothalamus. TSH and TRH concentration in circulation are regulated by T_3 and T_4 concentration; thyroid and TSH concentrations are regulated by iodide concentration in the blood stream obtained during daily intake [2,3,22,23,24,25].

TSH mainly activates the AMPc route which stimulates transcription factors (CREB, TTF-1 and -2, PAX8) and culminates by activating the transcription and expression of molecules implicated in T_3 and T_4 hormone synthesis (i.e. NIS in basal membrane, Tg in RER and its exocytosis, TPO and Douxs in the apical membrane and H_2O_2 formation). TSH's effect can be shown by increased T_3 and T_4 in the blood stream [26,27].

Normal iodide in circulation ranges from 10E-9 to 10E-7 M. Concentrations of this ion greater than 10E-5 inhibit T_3 and T_4 organification and synthesis during the first 48 h (called the Wolff-Chaikoff effect) [1], regardless of TSH concentration. Iodide organification inhibition directly depends on iodide intrathyroid accumulation [28]. This thyroid auto-regulatory effect happens when inorganic iodine concentration in blood exceeds a set threshold (overload) and the gland blocks iodine's organic binding for 48 h [1]. The gland

adapts once such 48 h have elapsed and organified iodide escapes, producing new hormones [29]; NIS expression becomes reduced at this time, as does iodide capture [30]. It has been suggested that there is a reduction in the function of the molecules implicated in organification and hormone formation: TPO, Duox 1 and 2, pendrin and Tg [30,31]. Thyrocytes in culture in the presence of 10-E3 M iodide reduce NIS expression and inhibit TPO and Tg synthesis [32]. Such reduction of NIS does not happen in hypothyroid mice, nor is Duox 1 and 2, TPO, pendrin and Tg gene expression modified [33]. An excess of iodide leads to iodide organification inhibition depending on TPO and not on NIS. TSH effects become reduced in the presence of strong concentrations of iodide, resulting in them adopting antagonic roles [34].

2. Thyroid culture

2.1. Introduction

Thyroid tissue fragments were kept on glass in saline solution, or *in vitro* (as this involved a glass vessel). Established the neuron cell theory, it has since been established that the unit of life is a cell (i.e. cell theory 1910) and cell culture or *in vitro* study began [35]. Cell cultures were then developed, thereby leading to studying cell functions in controlled conditions and different descriptions of culture mediums, supports and conditions have been developed from 1910. Fibroblasts in culture leave a matrix on culture surface on which endothelium cells from blood capillaries can be cultured; this has been called an extracellular matrix (ECM) [36]. Extracellular supports close to the ECM surrounding cells *in vivo* (such as collagen, laminin, fibronectin or matrigel) are currently being used [37].

New culture techniques were developed in 1975 in view of the close structure-function relationship, recognising the importance of organs' functional units, such as isolating and culturing isles of Langerhans from the pancreas [38] or "acini" regions from the lactant mammary gland or epithelial structures which needed to be conserved in polarised cell culture [39]. Thyroid follicle incubations and cultures could also be mentioned here.

A brief description of the most pertinent techniques for culturing the thyroid, isolated thyrocytes and/or thyroid follicles is given below.

2.2. Organotypical culture or organ culture

Organ culture or organotypical culture consists of culturing an organ's fragments or explants. Regarding the thyroid, this began with 2 to 3 h incubations (the term usually used to refer to cultures lasting less than 24h) of sheep thyroid fragments in the presence of radioactive iodide thereby demonstrating *in vitro* the ion's incopoproportion into diiodinethyronine (DIT) and T_4 [40]. When the transmission electron microscope was developed in the 1970s, this led to an ultra-structural description of thyrocytes *in vitro*; the first descriptions of thyrocytes' morphological changes in different culture conditions were made. It was shown that organ culture thyrocytes had reduced RER and GC in the absence of TSH [41,42]. Thyrocyte follicular architecture and ultra-structure were rapidly lost in

some of the models which were described. Approaching the 1990s attempts were made to use very small fragments (less than 1mm^3) in organ cultures (called mini organ cultures) which lasted 2 to 3 days without necrosis, exhibited iodide, sulphate and phosphate transport, synthesised a 19S Tg (normally glycosylated and iodised) [43] and were maintained for up to 7 days without cell death when coated with collagen [44]

2.3. Isolation and monolayer culture or cell culture

Monolayer culture (better known as cell culture) mainly deals with a single cell type. This implies tissue dissociation by enzymatic digestion or mechanical action and the isolation of cellular types by different sepaproportion methods. Isolated cells are placed on different types of supports where they adhere and proliferate in a single layer until reaching confluence (called primary culture). Secondary culture consists of sowing cells removed from the primary culture in fresh recipients and so on. The term passage is used to indicate the number of successive secondary culture sowings, thus the 1st secondary culture is the 1st cell passage. Thyrocytes were first cultured in 1911 [45]. Using this dissociation technique and continuous shaking during culture has shown that sheep thyrocytes concentrate radioactive iodide and incorporate it in iodine-thyronine: MIT, DIT and T4 [46]. Isolated and small cells, aggregates of 10 to 15 thyrocytes, are obtained after dissociation with trypsin [47,48]. One of the greatest drawbacks is the loss of cultured thyrocytes' cellular polarity when one wishes to study thyroid physiology since such polarity is fundamental in conserving thyrocyte membrane domains, and thus the expression of domain-specific molecules guaranteeing hormone synthesis [49].

Thyrocyte cultures were developed in dual chambers during the 1990s on cubic monolayers as *in vivo* with binding complexes in the lateral membranes' apical region, separating in thyrocytes' the apical membrane domains from the basolateral membrane domains, TSH favouring such cellular polarisation [50]. This model has demonstrated that ion flow is determined by thyrocytes' polarity, thereby corroborating the fact that ion channels are different in both thyrocytes membrane domains when thyrocytes' cubic form is conserved. A new channel has been described for the thyrocytes' apical membrane [51]; this new channel is CLC5 which is located in the apical region *in vivo* and it has been proposed that thyrocytes have a position in the apical membrane for controlling pendrin, the I$^-$/Cl$^-$ transporter [15].

The foregoing has shown the importance of conserving cell polarity and cubic form in thyrocyte culture for studying the gland's physiology and biochemistry.

Cell lines are continually growing and indefinitely proliferating cell cultures because they have lost control over their own cell division, contrary to primary and secondary cell cultures which die after a finite number of passes or subcultures, as is genetically determined in normal cells. Thethyrocyte cell line was described [52,53]; Fischer rat thyroid cell line or FRTL is most used around the world as it has a more similar ultra-structure to thyrocytes and synthesised Tg. These have been very useful in studying gene expression, cytoskeleton modification with different factors, iodide flow and that of other ions.

Such studies have led to advances being made in knowledge regarding some precise processes but there are limitations for extrapolating this to the gland *in vivo* because they are cells which lost certain control over their tissue of origin.

2.4. Pseudofollicles

Some of the main problems involved in thyroid physiology *in vitro* studies are the loss of follicle architecture, thyrocyte polarity and T3 and T4 hormone synthesis [54]. As mentioned above, the follicle lumen disappears in the monolayer with its colloid and the thyrocyte membrane domain polarity necessary for carrying out hormone synthesis [55,56]. However, tridimensional structures can be induced by covering monolayers with ECM elements, they become re-organised into two- to four-cell structures around the intercellular cavity in the presence of TSH, called pseudofollicles by some authors and "follicles" by others [54,56,57,58,59,60,61,62]. These pseudofollicles are unstable, short-duration structures and do not reproduced *in vitro* the function of iodide incorporation in Tg or synthesis of T_3 or T_4, and shown that follicle structures' thyrocyte polarity is necessary for studying thyroid physiology and the molecular processes implicated in such function [63].

3. Follicles

A new culture technique was developed from 1965 to 1980 where the functional unit of different epithelial organs, isles of Langerhans in the pancreas [64,65] or the acinar region of the mammary gland [39] are isolated and cultivated, conserving *in vivo* morphology.

It is clear that conserving multicellular structures *in vitro* forming exocrine glands' functional units depends on preserving apical-basal polarity for imitating *in vivo* functions; this is why attempts at characterising the factors generating polarity and which molecules allow maintaining 3D structures *in vitro* in mammary gland acini [66], the endocrine pancreas [67] or kidneys [68] are continued.

The 1980s saw the beginning of 24-h thyroid follicle cultures [23,69,70,71,72,73,74] on agarose, to avoid cell adhesion and monolayer formation [75]. Such incubations showed that iodide organification and H_2O_2 production took place in colloid [23,72] and that rat thyrocitos of open and closed follicles incubated for 12 h on agarose conserved their apical-basal polarity and *in vivo* ultra-structure, in basal region nucleus surrounded by RER, supranuclear GC, in apical region vesicles' and microvellosities. As well as responding to TSH-forming pseudopods [16,23,75], synthetic TSH peptides bound in the basolateral membrane [76]. Closed follicles were maintained for up to 3 days [69] and responded to thyrocytes' TSH, increasing RER [23]. TSH has stimulated pig and human open follicle thyrocyte function in culture during the first two days [49,74]. Pig open follicles cultured for 48 h with forskolin have been used for determining the function of H_2O_2 formation which is important in hormone synthesis [77]. The presence of TSH- or forskolin-induced cyclic adenosine monophosphate (AMPc) route stimulants has been seen to be indispensable in these cultures and has reiterated the importance of conserving follicle structure in culture

for promoting hormone formation, as happens with thyroid gland apical membrane *in vivo*. It is so important that specific genes have been described which govern follicle formation and maintain follicle architecture, the gene transcribing the thyroid-specific enhancer-binding protein (T/ebp or Nkx2.1) regulating the transcription of genes implicated in hormone synthesis: NIS, TPO, TSH receptor and Tg and re-organised transfecteds thyrocites in follicles [78]. Human goitre follicle structure has been covered with collagen to preserve it longer and cavities in human [79,80] and mouse thyrocyte [78] thin cell bilayers have persisted 2 days more. Collagen's importance in preserving epithelial cells' apical-basal polarity has been described for obtaining MDKC (Madin-Darby canine kidney epithelial cell line) cell 3D cultures, but hepatic growth factor (HGF) was added to cultures [81].

The methodology developed by our group for reproducibly obtaining closed follicles, conserving their architecture and function in culture analogously to that of the gland *in vivo* is described below.

4. Method used

Most of the first work on rat follicle culture was open and became disorganised during the first days of culture [16,23,72]. We based our approach on these rat thyroid dissociation techniques. We describe the importance of isolating closed follicles, their culture and long-term response to TSH and iodide (9 and 12 days) and to increasing doses of iodide. Details are given of the isolation methodology, the morphological study of these isolated follicles and in culture at morphological level by inverted (IM) optical (OM), electron (TEM) and (CM) confocal microscope and their functional study: iodide accumulation and organification, Tg, T_3, T_4 synthesis and NIS localisation.

4.1. Isolation and closed follicle culture

Wistar rat (200g) and ICR mouse (30g) thyroid was used; the animals were obtained from the Universidad Nacional de Colombia's Bioterium. Pig Cialta and strain 769 thyroid was provided by two slaughterhouses in Bogotá. Some having sub-clinical hyperthyroidism due to β- energetic injection, having T_3 (1.34 ng/dL) and T_4 (107.0 ng/dL) within the normal range and excessively low TSH (<0.005 mUI/mL), were called hypothyroidic, as morphologically and functionally described in mice [33], whilst the others were called euthyroidic. The animals were handled according to Colombian considerations for animals being used in research and care of animals for domestic consumption.

The methodology mainly involved rat thyroid and was corroborated in mouse thyroid. Obtaining human thyroid fragments is difficult; pig was thus used due to its similarity with human metabolism [82,83,84,85,86], even though it is hoped to begin cultures with human thyroid in the near future. The differences between rodents and pig had to be considered. General metabolism regulated by the thyroid gland in rodents is 10 times greater than that in pigs and humans. Follicle diameter ranges from 50 to 150 μm in rodents, whilst this is 150 to 500 μm in pigs and humans. Rodent lobes range from 3 to 5 mm^3 at their widest whilst

this is 3 to 5 cm^3 in pigs and humans, meaning that many rodents must be sacrificed for each experiment; 30% to 40% of a pig's lobe is used. Rodents' capsule is thin and the parenchyma does not have large amounts of connective tissue; this capsule is thick in pigs and connective tissue septa are very abundant and extensive. Shaving razors are used for stereoscopic micro-dissection. The capsule of rat and mice lobes is eliminated; each is cut in two along its major axis whilst pig lobes are opened in two with a scalpel and cut into 7 to 10 mm^3 fragments. Connective tissue is then eliminated as far as possible using stereoscopic microdissection with razors without affecting the parenchyma. Around 3 mm^3 fragments are obtained (similar to rodent fragments) without connective tissue visible by stereoscope.

The thyroid fragments are put together and washed 3 times with COON medium [55], enzymatically dissociated with collagenase II which digests collagen (250 U/mL rodents; 400 U/mL pig) and 2 µg/mL DNase 1 (nb, the original article [82] read "2 mg/ml DNase I" when it should have been 2 µg/ml). Dead cells form aggregates which are avoided with DNase which only dead cells' DNA digest and become fragmented; live follicle cells or fragments become attached to these aggregates if DNase is not added [23].

Thyroid fragments become dissociated in enzyme solution in COON medium at 37ºC and being shaken at 140 oscillations per minute; without delay, they are mechanically dissociated in this solution, aspirating and expelling enzyme solution containing thyroid fragments with 20 mL pipettes (3 to 5mm distal diameter; extreme for liquid entry and exit from pipettes), 10 times. The technique with rodent fragments continues with 10mL pipettes (1.5 mm distal diameter), 10 times. Such pipette dissociation is done at 10 min intervals during enzyme dissociation (i.e. the supernatant containing isolated follicles is collected every 10 min after pipette dissociation and fresh enzyme solution added for the following 10 min). This is done three times x 10 min with rodent thyroid and 6 x 10 min with pig thyroid; this is a modification of already described dissociation [16,23,72] and is most important for avoiding follicle opening. Most follicles are isolated during a second dissociation for rats and mice and in a third and fourth for pigs.

The follicles isolated during each 10 min interval are washed 3 times with COON + 2% foetal calf serum (FCS) spun at 50g for rodent follicles and 30g for pig follicles. This must be done in a free rotor centrifuge using low centrifugal force, otherwise centrifugal pressure opens up many follicles. All the follicles are placed together and filtered through 100 µm pore diameter mesh for rodents; those for pigs are left to decant at 1g for 10 min. Dissociation pre-incubation or recuperation time is continued for 4 h for rats and mice and 12 h for pigs in COON medium + 0.5% FCS in a 95% air - 5% CO$_2$ atmosphere and 100% humidity on 1% agarose type I (less grouping than with agarose type II) to avoid cells adhering to the support [44,75]. Culture medium (the same as pre-incubation) is changed for aspiration with follicles; it is spun at 50g for rodents and 30g for pigs. The supernatant is skimmed off and fresh medium added to begin culture in the same ambient conditions and on agarose type I; the same is done for changing medium when making the culture.

Undissociated fragments (around 0.8 mm^3) remaining after enzyme dissociation are washed 3 times with COON + 2 % FCS and cultured in the same conditions as for follicles but with 2% FCS. This has been called mini organ culture, according to Bauer and Herzog [43].

Different supports have been tried for maintaining follicular architecture, such as glass, plastic, collagen or collagen coated (1mg/mL), and 1%, 5% and 10% FCS concentration. Closed follicles are conserved in culture; however, thyrocyte monolayers grow proportionally to serum concentration in the medium. Monolayer growth should be avoided because this increases iodide accumulation values and interferes with analysis of iodide organification function; fresh enzyme dissociation must be carried out to recover the follicles [82,86]. When follicular cell fragments are cultured in collagen they do not reform follicles as has been described for human [79,80] and mouse thyrocytes [78]; some MDKC epithelial cells are organised in follicle-like structures requiring HGF [81]. The foregoing meant that the use of glass, plastic and collagen for follicle culture was rejected and agarose type I used instead.

Follicles' functional and morphological state was controlled before beginning the cultures by 5% Trypan blue exclusion exam [87], cell viability was determined by IM which also allowed visualising open or closed follicle architecture. Thyrocyte viability is usually around 100% immediately after isolation and before beginning pre-incubation. Cells which do not exclude Trypan blue are usually endothelium cells bound to follicle periphery (Figure 3B solid black arrows) or follicular fragment aggregate thyrocytes (Figure 3B circle).

Iodide accumulation and organification is determined (5 µCi/mL Na^{125}I, 4 h) for ensuring a high percentage of closed follicles; the importance of this control before beginning culture is described and discussed ^{125}I in 6.1. Importance of obtaining closed follicles.

Follicles are culture for 0, 1, 3, 6, 9 and 12 days with TSH and without TSH (1 and 0.1 mU/mL rat; 1 mU/mL pig) in the same conditions as for pre-incubation. Culture medium is changed during these days aspirating it with the follicles and spinning at 50g for rat and 30 g pig, discarding the supernatant which usually contains cell and follicle fragments. The follicles are examined by IM during each stage. 5 µCi/mL Na^{125}I is added each culture day 4 h before collecting the follicles for morphological and functional studies.

The follicles are cultured for 1 day with and without TSH (0.1 mU/mL rats; 1 mU/mL pigs) for studying the effect of different iodide concentrations. The medium is changed and the follicles cultured for 0.5, 3, 8, 12, 24 and 48 h with 10E-10, 10E-7, 10E-5, 10E-3 M Na^{127}I and Na^{125}I 5 µCi/mL (kindly donated by Manuel E Patarroyo) with and without TSH. The follicles are collected after such treatment for morphological and functional analysis.

4.2. Morphological studies

Morphological study involves impregnating follicles in Epon resin; follicles are spun at 300g for dehydratation before being impregnated in the resin [23,82,83]. Semi-fine, autoradiographed slices are observed by OM and ultra-fine slices by TEM.

Protein synthesis and NIS expression reduce excess iodide [30,31,32] and (bearing in mind that NIS has not been described in closed follicle cultures) the presence of NIS is determined in culture in the presence of different iodide and TSH concentrations, with anti-NIS/GS antibodies (1:500, kindly donated by Thierry Pourcher) and Alexa 488 anti-rabbit secondary

antibodies (1:1,000). The nuclei are visualised using DAPI/PBS (1:9,000). Follicles are compressed on commercial laminas as their diameter is greater than that of cells; a 1 mm high chamber was constructed to enable observing by CM without follicular compression.

4.3. Functional studies

Iodide organification (which is essential in thyroid hormone synthesis) becomes lost during the first days in the thyroid culture models described to date. Functional analysis of follicles in culture for determining whether follicular architecture is conserved has advantages over other cultures regarding hormone synthesis.

Follicles' accumulated and organified ^{125}I radiactivity is determined by γ well counter. Rat follicles are washed 3 times for 5 min at 50g and at 30g for pig follicles with COON + Na^{127}I (cold) 100 times the concentration of radioactive iodide used in culture and the radioactivity arising from accumulated (A) is determined. 10% trichloroacetic acid + Na^{127}I 100 times the radioactive iodide concentration used in culture is then added and the radioactivity present in protein precipitate corresponding to protein binding iodide (PBI) or iodide organification (O) is determined.

The precipitate is used for determining the amount of DNA by diphenylamine method [88], Tg19S by HPLC and iodine-thyronine (MIT, DIT, T$_3$, rT$_3$ and T$_4$) in Tg by inverse-phase HPLC [89]. The results are expressed in % iodide dose in μg DNA. Follicle structure does not allow the number of cells to be counted and statistical analysis requires having a parameter letting the results be homogenised; based on 1 pg DNA/mL equals 2E5 cells, the number of cells present in cultures can be determined and the results statistically correlated [82].

4.4. Statistical analysis

Data given in the text are expressed as mean standard deviation for N values. Significant differences are established between some times for A and O variables by Student's t-test.

5. Results

Closed follicular architecture is indispensable in agarose culture since open follicles become dissociated and cells die; it is thus essential to begin with maximum closed follicles possible to avoid this. The following item gives the criteria determined important for beginning culture. Then, the long-term culture results in which closed and isolated follicle morphology and function were compared. Last, the morphological and functional results in the presence of different doses of iodide and TSH.

5.1. The importance of obtaining closed follicles for culturing them

Many isolated cells and follicular fragments were obtained when thyrocytes became detached after 30 contiguous min, instead of 10 minute enzyme dissociation time (Figure

3A); these thyrocytes were eliminated 24 h later when culture medium was changed. If some fragments persisted after 1 or 2 days' culture they became dissociated and thyrocytes died because they could not adhere to the agarose covered culture support [75] since normal cells require support for growing in culture. Trypan blue allowed an approximation of follicles'
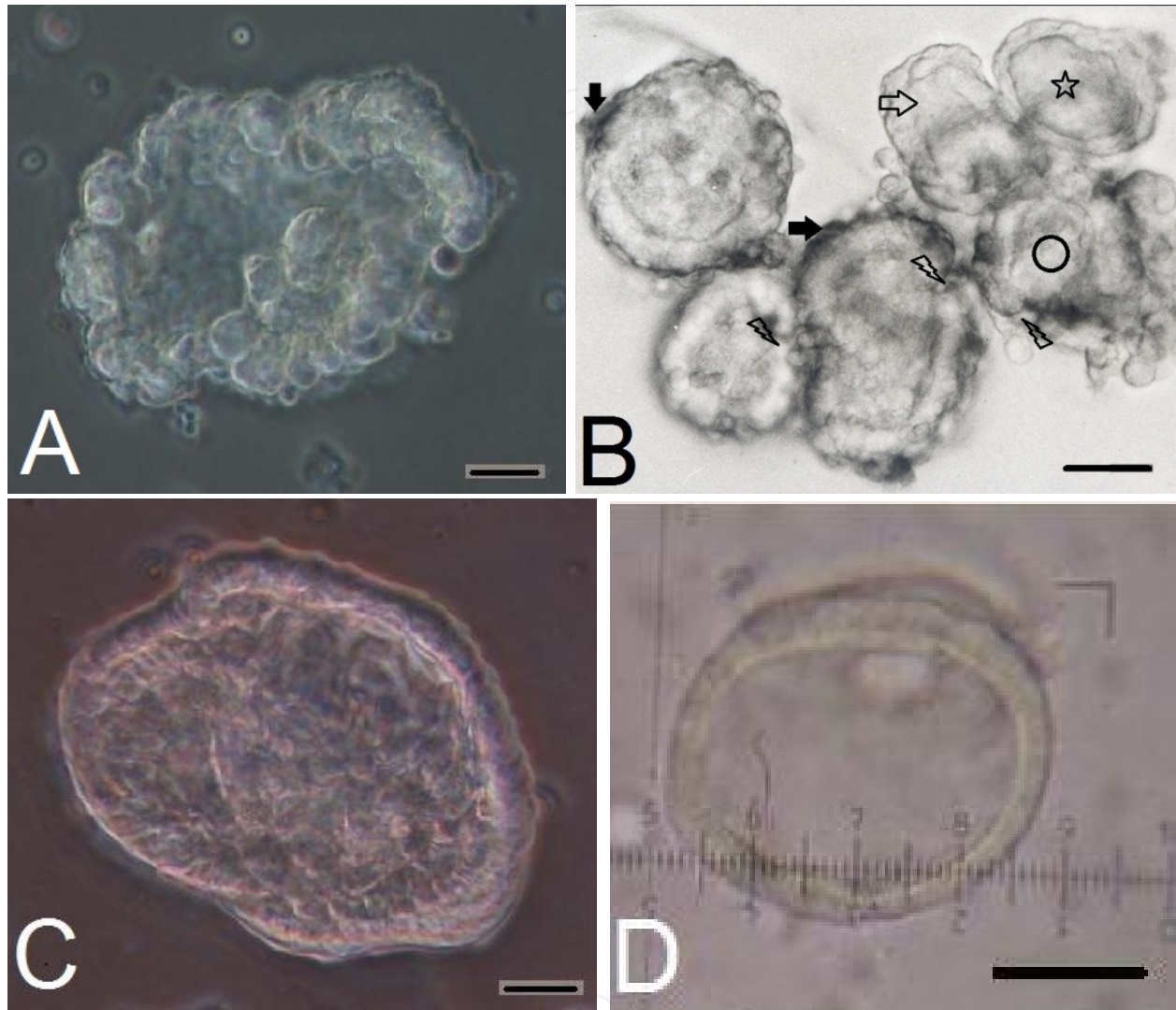

Figure 3. A. A follicle fragment from euthyroidic pig thyroid dissociated for 30 min without interruption pre-incubated for 12 h. Note the contour of thyrocytes which became detached from the follicular fragment and cell waste in culture support. **B.** Trypan blue for recently isolated rat thyroid follicles by strong dissociation (Strong dissociation, Table 1). Follicles which did not open (star) conserved colloid birefrigence and a clear and continuous boundary between colloid and cells, whilst those which became resealed (clear arrow) lost colloid birefrigence and the boundary between cavity and cells was not clear. Openings could be seen in those which did not reseal (triangles beam). Cells which did not exclude stain were mainly endothelium cells found in follicle periphery (solid arrows). Follicle fragments presented non-viable thyrocyte aggregates (circle). **C.** Pig euthyroid resealed follicles 12 h pre-incubation. Note that the thyrocytes' apical boundaries could be distinguished due to a lack of colloid. **D.** Trypan blue of closed follicle with colloid birefrigence of pig hypothyroids pre-incubated for 12 h; colloid was birefrigent and the boundary between colloid and thyrocytes was clear and continuous (IM. Scale bar: A 20 μm, B 50 μm, C 15 μm, D 70 μm).

closed or open state (Figure 3B); those conserving colloid showed up due to birefringence in IM and there was a clear boundary between colloid and cells (Figures 3B star and 3D) whilst those which became resealed lost their colloid birefringence and the boundary between cavity and cells was not clear (Figures 3B clear arrow and 3C). Openings in follicles which did not become resealed appeared (Figure 3B triangles beam) as did colloid loss. Cells which did not exclude the stain were mainly endothelium cells which were found on follicle periphery (Figure 3B black arrows). Follicle fragments could be seen because they lost follicular structure and organisation continued from thyrocytes' epithelial layer (Figure 3B circle).

Follicle morphology and function were analysed after 4 hours' labelling with radioactive iodide, varying according to the dissociation procedure used with and without pre-incubation. If pipette dissociation after each 10 min enzyme digestion was done in such a way that hydrodynamic forces were produced with turbulence, this was called **strong dissociation,** but when this was done slowly without turbulence in the liquid and avoiding air-bubble formation it was called **mild dissociation**.

Iodide which did not bind to molecules was eliminated in fixation liquid until being impregnated in resin; labelling in autoradiographs (following pre-incubation, added for 2 h in the presence of $Na^{125}I$ 5 µCi/mL) was that which bound to proteins and was equivalent to organified iodide. The number of closed follicles could thus be counted and distinguished from open ones by autoradiographs. Open follicles were numerous in strong dissociation; they could be seen because no organified iodide was concentrated within follicle interior (Figure 4A star) even though histological cross-sections seemed to suggest that the cubic epithelial layer continued (Figure 4A circle). There were much fewer open follicles if dissociation was mild (Figure 4B). Follicles which resealed could be distinguished because, even though the labelling was homogeneous, it was less intense in colloid than in those which remained closed (Figure 4B arrow).

Pre-incubation time was another key aspect for obtaining a maximum of closed follicles at the beginning of culture (4 h for rat follicles and 12 h for pig follicles when this was divided into 4 initial hours in which medium was changed by aspirating the medium with follicles and spinning at $30g$ x 5 min followed by pre-incubation for 8 h). The pre-incubation period was essential since this time allowed cells to recover from the aggression of the enzyme used for dissociation.

Pre-incubation time following dissociation promoted an increase in closed follicles with two dissociations (strong or mild, 33% and 46%, respectively, Table 1). Open follicles which did become resealed or follicular fragments (Figure 4A) became disorganised and were discarded in the supernatant when changing the medium and spinning to wash them.

Morphological modifications included modification of the amount of iodide accumulated and organified by follicles (Table 1); if dissociation was mild, iodide accumulation (A) and organification (O) values and O/A percentage increased following pre-incubation time, whereas if dissociation was strong then these values were lower.
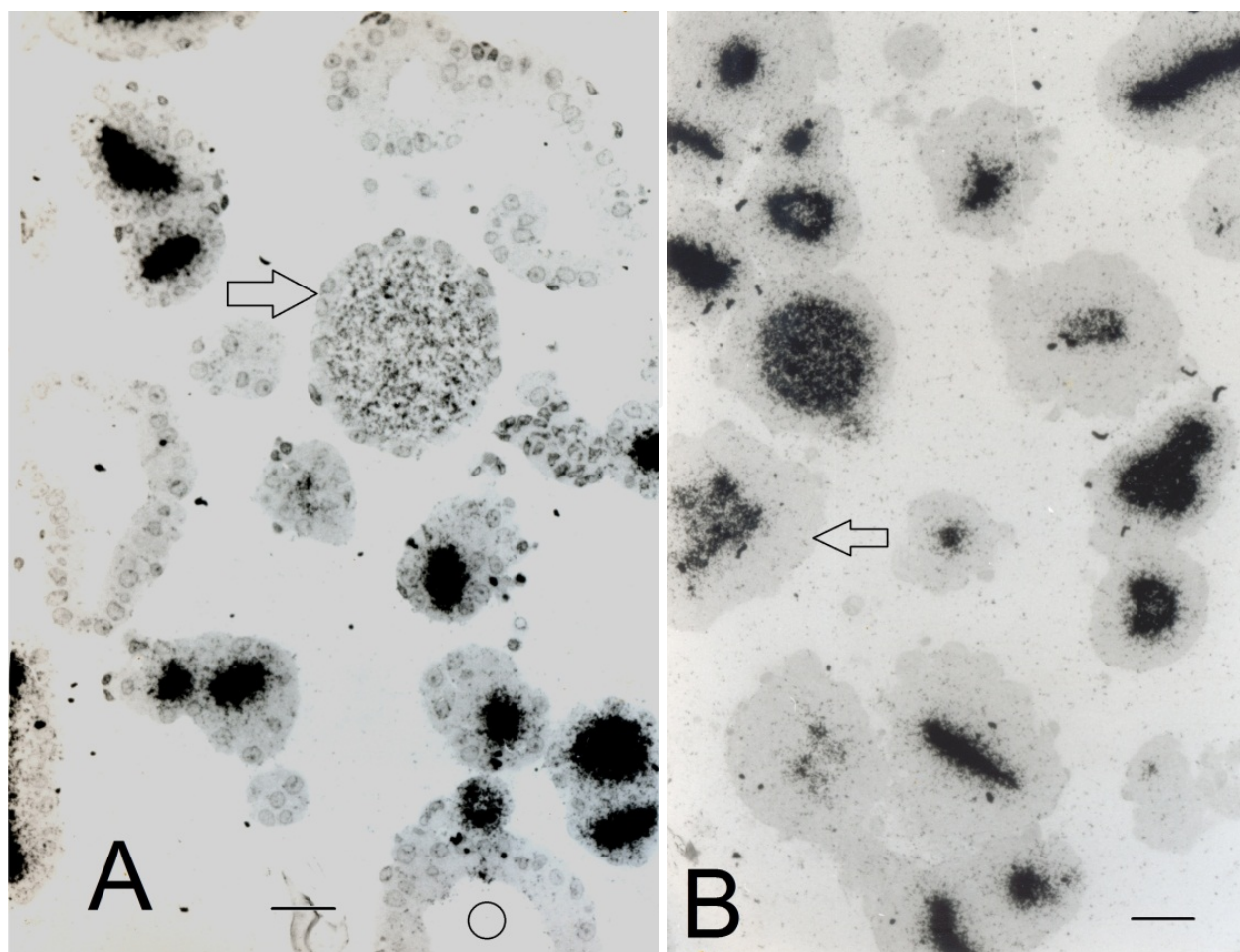
Figure 4. Autoradiograph of dissociated follicles (following pre-incubation, they were added for 2 h in the presence of 5 µCi/mL Na^{125}I). Iodide ions which did not bind to proteins were eliminated while washing the histotechnic preparation until being impregnated in resin. Closed follicles were labelled in the follicle centre even though they became resealed (arrow). **A.** Follicles isolated by strong dissociation (aliquot of follicles from Table 1, strong dissociation with pre-incubation). Follicles seeming to be closed by histological cross-section but which did not accumulate organified iodide in colloid were open follicles (circle). **B.** Most follicles isolated by mild dissociation (aliquot of follicles from Table 1, mild dissociation with pre-incubation) were closed because of intense labelling within follicular cavities (OM. Toluidine bleu. Scale bar: 25 µm).

Closed follicles counted on autoradiographies corresponded to O/A proportion percentage; thus mild dissociation gave 83% closed follicles and 85% O/A proportion whilst strong dissociation gave 45% closed and 58% O/A proportion (Table 1).

Follicles isolated by mild dissociation, following 2 h with radioactive iodide, accumulated iodide 4.8 times and 7.8 times more with 0.1 and 1 mU/mL TSH, respectively, regarding without TSH. TSH did not modify morphology regarding follicular architecture or O/A proportion (Table 2).

This follicle isolation method can be applied to other thyroid tissues from other species, such as rabbits or humans. The percentage of open follicles was greater in pig or human thyroid follicles and more tissue was obtained per experiment; 12 h was thus allowed for pre-incubation (Figures 4C and 4D).

Digestion conditions	% follicles closes	A	O	% O/A
Without pre-incubation				
Mild dissociation	57 ± 1,4	1.14 ± 0,01	0.89 ± 0,01	78
Strong dissociation	45 ± 5,7	1.84 ± 0,37	1.08 ± 0,14	58
With pre-incubation 4 hours				
Mild dissociation	83 ± 1,4	1.93 ± 0,24	1.64 ± 0,17	85
Strong dissociation	60 ± 7,1	1.32 ± 0,17	0.91 ± 0,01	68

Table 1. Influence of dissociation conditions and pre-incubation time on the percentage of rat thyroid isolated and closed follicles and their function. It can be seen that the percentage of organified iodide on accumulated iodide (O/A) was equivalent to the percentage of closed follicles concentrating radioactive ion determined by follicle count using accumulated grains in follicular colloid in autoradiography of semi-fine cross-sections (Figure 4). Average values for two culture dishes ± SD. Following pre-incubation, $^{125}I^-$ accumulation (A) and incorporation in proteins (O) were determined following 2 h in the presence of $Na^{125}I$ 5 μCi/mL and expressed in μg/dose/μg DNA.

TSH	A	O	% O/A
None	0.73 ± 0,18	0.58 ± 0,11	79
0,1mU/mL	3.48 ± 0,17	2.78 ± 0,04	80
1mU/mL	5.71 ± 0,72	4.98 ± 0,67	87

Table 2. Influence of TSH on iodide accumulation and organification in isolated rat follicles expressed in μg/dose/μg DNA. Follicles were cultured for 2 h in the presence of $Na^{125}I$ 5 μCi/mL immediately following pre-incubation. TSH was stimulated by $Na^{125}I$ but O/A proportion was around 80% with or without TSH. The value without TSH represented the percentage of closed follicles obtained following correct mechanical dissociation and pre-incubation of follicles. Average values for culture dishes ± SD (these values were representative of 3 experiments).

A good approximation of the percentage of closed follicles following pre-incubation must thus be born in mind (this corresponded to day 0 in our cultures). It can thus be generalised that O/A proportion values should be greater than 80% before beginning culture (day 0, Table 2) and that there should be a potentially high number of closed follicles, even though follicle diameter may vary in each species or come from different thyroid functional states [85,86,90]. Open follicles' ability to reveal themselves during the course of pre-incubation probably depended on the degree of initial opening. Even though groupings of rat thyroid cells became organised in the presence of TSH and on agarose, they became reorganised into 6- to 10-cell follicles which could be cultured for 3 days [69,73]. In our results, rat or pig follicles which became resealed did not require TSH for conserving their follicular architecture.

5.2. Long-term closed follicle culture

5.2.1. Functional study

On **day zero** (4 h pre-incubation in the presence of radioactive iodide) follicles accumulated 3.5 ± 2.1% of the dose in the medium **without TSH** (9 experiments in rats). Organified iodide percentage varied according to closed follicle percentage at the start of each experiment.

O/Aprioportion was 57% in experiment 1 and 91% in experiment 2 (Table 3); more than two thirds of organified iodide bound to stable Tg19S (Table 4), the rest of the molecules having lower molecular weight. Capture increased in the presence of **TSH** regarding without TSH $250 \pm 210\%$ (9 experiments in rats), but O/A percentage remained constant. The effect of TSH was evident on A and O, and higher than values without TSH (Tables 2, 3 and 4). Iodide accumulation on Tg19S slightly increased with TSH, but not significantly so (Table 4).

	Exp. 1			**Exp. 2**		
TSH	0.0	0.1	1.0	0.0	0.1	1.0
Culture, day						
0	57	46	69	91	93	94
1	69	75	71	78	94	93
3	64	85	85	84	86	87
6	0	58	83	60	51	84
9	2	51	71	59	78	91
12	0	54	82	50	73	90

Table 3. Evolution of ^{125}I$^-$ organification/accumulation in rat follicle culture. Comparing two experiments where rat closed follicle percentage varied during the course of two experiments (Exp. 1 and Exp. 2). 80% of follicles were closed at the start of culture counted in autoradiographs of culture aliquots in experiment 2 (*cf* 65% in experiment 1). Na^{125}I 5 µCi/mL was added 4 h before collecting follicles during each day of the experiment, expressed in %/dose/µg DNA. Each value was the average of two samples or culture dishes. Experiment 2 was representative of 7 independent experiments. TSH: mU/mL.

Following **one day**'s culture without TSH, accumulation was low at the start of culture; it did not become modified, but if it was high it became reduced, whilst incorpoproportion of iodide in Tg became reduced (Table 4). T_3 and T_4 proportion in regarding Tg was not modified. Iodide accumulation increased in the presence of TSH, O/A percentage remained high and in starting values (Table 3). Iodide incorpoproportion in Tg19S was better (Table 4) and Tg19S content in follicles did not vary.

Accumulation fell abruptly on the **third day** without TSH when starting with a low percentage of closed follicles, just like O/A percentage, whilst it became reduced with a high percentage of closed follicles, but O/A was maintained. O/A values were high in the presence of TSH and remained higher than 80% (Table 3).

Accumulation and organification values and their O/A percentage differed on the **sixth day** regarding closed follicle percentage; A and O could not be determined in experiment 1 which began with 57% (Table 3. Exp. 1) whilst experiment 2 began with 94% and became reduced to 60% (Table 3. Exp. 2). There was a reduction in all O/A percentages in rat or pig cultures by the sixth day which also differed with the percentage of closed follicles (Table 5), animal species and functional state at the start of culture. The percentage of iodide on Tg19S also became reduced (Table 4).

		A		^{125}I$^-$ in	Tg19S*	
TSH	0.0	0.1	1.0	0.0	0.1	1.0
Culture. Day						
Without NaI						
0	5.8	5.5	7.2	59	63	67
1	1.4	2.7	3.1	43	66	65
3	1.3	4.5	8.7	46	50	52
6	0.7	1.2	4.0	25	13	42
9	0.2	0.6	4.0	16	17	55
12	0.2	0.7	8.0	15	25	52
With NaI						
0	5.8	7.8	9.3	60	61	66
1	1.1	1.8	3.1	31	58	64
3	0.7	4.2	7.6	49	49	52
6	0.1	1.4	3.4	10	22	39
9	0.2	1.6	2.6	14	32	38
12	0.1	1.9	2.9	11	33	58

Table 4. Evolution of iodide accumulation (A) and its incorporation in stable Tg19S in rat follicle culture. Effect of adding a small dose of cold iodide (10E-10 M Na^{127}I). A was expressed in %/dose/µg DNA. Radioactivity determined in stable Tg19S was expressed in % ^{125}I$^-$ accumulation/dose/µg DNA. The results without NaI were those expressed in experiment 2, Table 3. Na^{125}I 5 µCi/mL 4 h was added before collecting follicles during each day of the experiment. Each value was the average of two samples or culture dishes.

O/A retained the same values on **day 9 and 12** as those for day 6 in both experiments without TSH, whilst with TSH this increased in experiment 1 regressed to day 1 values and then maintained similar values to those of the two first days. The percentage of iodide on Tg19S with and without TSH was conserved (Table 4).

The presence of a small dose of iodide (10E-10 M NaI) did not modify iodide accumulation values at the start of culture (Table 4), but responded better to TSH. Tg19S had more T_3 and T_4 and TSH increased the percentage of T_3. O/A percentage increased with 0.1mU/mL TSH on days 6, 9 and 12 whilst it fell with 1 mU/mL (not significantly). Iodide had no effect on stable Tg19S content in follicles; more than two thirds of accumulated iodide was incorporated in Tg19S (Table 4) even in the absence of TSH where iodide accumulation was very low on day 12.

Preserving the function of pig follicle thyrocytes during culture also depended on the percentage of closed follicles since the beginning. However, this differed from those for rats in the absence of TSH, because this began with 43% O/A equivalent to the percentage of closed follicles presenting iodide organification on days 6 and 9 (Table 5, euthyroidic follicles) contrary to rat follicles which was zero (Table 3. Exp. 1). As 7 times more material was obtained from pig thyroids than rat follicles, a greater number of recovered follicles were conserved in changes of medium by centrifuging, or different species having variations in response to the same medium conditions.

| Culture, day | TSH | Euthyroid | | Hipothyroid | |
		% O/A	N	% O/A	N
0	0	43.4. ±.5.9	4	82.4. ±.7.2	4
	1mU/mL	61.5. ±.4.6	4	80.5. ±.3.7	4
1	0	56.94. ±.4.7	3	77.0. ±.3.8	4
	1mU/mL	71.4. ±.7.1	2	76.44. ±.3.9	4
3	0	37.4. ±.6.6	2	82.24. ±.4.1	3
	1mU/mL	43.2. ±.5.9	2	76.44. ±.5.3	2
6	0	9.16. ±.2.2	2	23.3. ±.9.4	2
	1mU/mL	15.0. ±.3.7	2	52.32. ±.4.2	2
9	0	52.66. ±.2.2	2	16.62. ±.1.5	3
	1mU/mL	64.8. ±.4.3	2	53.6. ±.5.0	3

Table 5. Evolution of $^{125}I^-$ organification/accumulation (O/A proportion) percentage for pig follicles. Hypothyroid follicles had high O/A percentage at the start regarding euthyroids (representative results from 4 experiments). This experiment began with less than 50% O/A in euthyroids for comparing with Table 3, experiment 1; however, when 3 experiments began with euthyroid follicles having 80% or more O/A they behaved like those for rat follicles in experiment 2, Table 3. 5 μCi/mL Na^{125}I were added 4 h before collecting follicles on each day of the experiment expressed in %/dose/μg DNA. Average values for the number (N) of culture dishes ± SD.

The difference between euthyroidic rat or pig follicles regarding hypothyroid ones was that hypothyroids responded to TSH on the sixth and ninth culture days, perhaps due to adapting to normal culture conditions, like hypothyroid glands' response *in vivo* when the effect induced by hypothyroidism stimulated with TSH was eliminated [24].

We compared isolated follicles' iodide accumulation and organification with their respective pig mini organ cultures lasting up to 9 days. Euthyroidic tissue became disorganised after the sixth day and functionality could not be determined after this day. Some follicles were conserved in the hypothyroids on the outside of the cultured fragment and presented this function on days 6 and 9 (Table 6). Mini organ culture functional values were higher than those of follicles isolated on the first day, but these were not significant; they were higher from the first day onwards in isolated follicles, maintaining higher values up to day 9 (euthyroidic and hypothyroid) (Table 6).

TSH	A	O	% A/O	N	TSH	A	O	% A/O	N
		Euthyroid, follicles					**Euthyroid, mini organ culture**		
0	0.049	0.025	51.6 ± 0.052	4	0	CND	CND		3
1mU/mL	0.074	0.048	65.3 ± 0.043	4	1mU/mL	CND	CND		3
		Hypothyroid, follicles					**Hypothyroid, mini organ culture**		
0	0.122	0.0633	36.75 ± 0.67	4	0	0,115	0.025	22.7 ± 1.5	3
1mU/mL	0.122	0.0655	53.6± 0.050	4	1mU/mL	0.723	0.278	38.5 ± 0.067	2

Table 6. Determining iodide accumulation (A) and organification (O) for isolated follicles and mini organ cultures from the same pig thyroids after 9 days' culture. 5 μCi/mL Na^{125}I was added 4 h before collecting follicles and mini organ cultures on each day of the experiment expressed in %/dose/μg DNA. Average μg of follicle DNA was 4.5 and 14.7 for mini organ cultures. Values represent the average number (N) of culture dishes ± SD. CND: could not be determined.

It has been described that pig thyroid mini organ cultures enables studying "thyroid tissue structural and functional integrity *in vitro* [54]"; however, we have considered that studying thyroids *in vitro* is better done with isolated follicles than using mini organ cultures. Since closed follicles maintain their architecture throughout culture time, thyrocytes are viable and their basement membrane is in direct contact with the medium and not with capillaries whose endothelial cells die rapidly in culture during the first 24 h (Figure CB).

DNA content per culture dish did not show a significant change during 12 days' rat follicle culture (1.55 ± 0.52 µg/dish, N = 16) or 9 days' pig follicle culture (5.35 ± 0.36 µg/dish, N = 25).

Even though closed and isolated follicles in culture had differences regarding stable Tg19S, the amount of T_3 and T_4 and iodide accumulation between different treatments with and without TSH and with or without 10E-10 M NaI, the follicles did have more iodide organification, iodised Tg19S and T_3 and T_4 at 12 days' culture, even without TSH [83,86], than in all other culture models published to date except of the group [83,84,85,86]. Such variations were homologous to glands *in vivo* in the same study conditions.

Our culture system has different characteristics distinguishing it from other models described up to now. Monolayers lose their function on the first day [48], become reorganised in pseudofollicles on the third day and only 2% to 4 % become incorporated or organified in iodine accumulated in poorly iodised Tg (Tg16S) [91], even though higher than 90% O/A with TSH has been reported for a matrigel-covered monolayer culture forming a double cell layer having cavities [61]. Different models mentioning culturing "follicles" [92,93,94,95] have not shown these functions in their results; others culturing pig "follicles" for 2 days, based on Björkman and Ekholm [16] as we, require 5% FCS, 1 mU/mL TSH and non-physiological molecules such as forskolin or 8-(4-chlorophenylthio)-cAMP for maintaining thyrocyte functions [77]. Using closed follicles enables functional parameters to be conserved and measured: iodide accumulation, iodide organification and, particularly, Tg19S equivalent to that *in vivo* and T_3 and T_4 formation throughout culture with or without TSH. We have also shown that maintaining closed follicular architecture is an indispensable condition for conserving such thyroid functions in culture *in vitro*. If follicular architecture is to be conserved, it is not enough to maintain functions at the same values as those at the start of culture as TSH is required and culture becomes improved by adding iodide, as thyroid function *in vivo* is governed by TSH and iodide.

5.2.2. Morphological study

Pig hypothyroid follicles cultured 1 and 3 days without TSH have a very thin epithelial layer (Figure 5A) like original tissue's follicle epithelium. The epithelium became cubic after day 6 and was preserved up to day 9 (Figure 5C). It was seen that follicles conserving colloid had a birefrigent aspect when observed by IM, thereby showing that this was conserved during culture. Epithelium thickness increased in the presence of TSH, becoming cubic on the first day of culture (Figure 5B) and being maintained so until day 9.
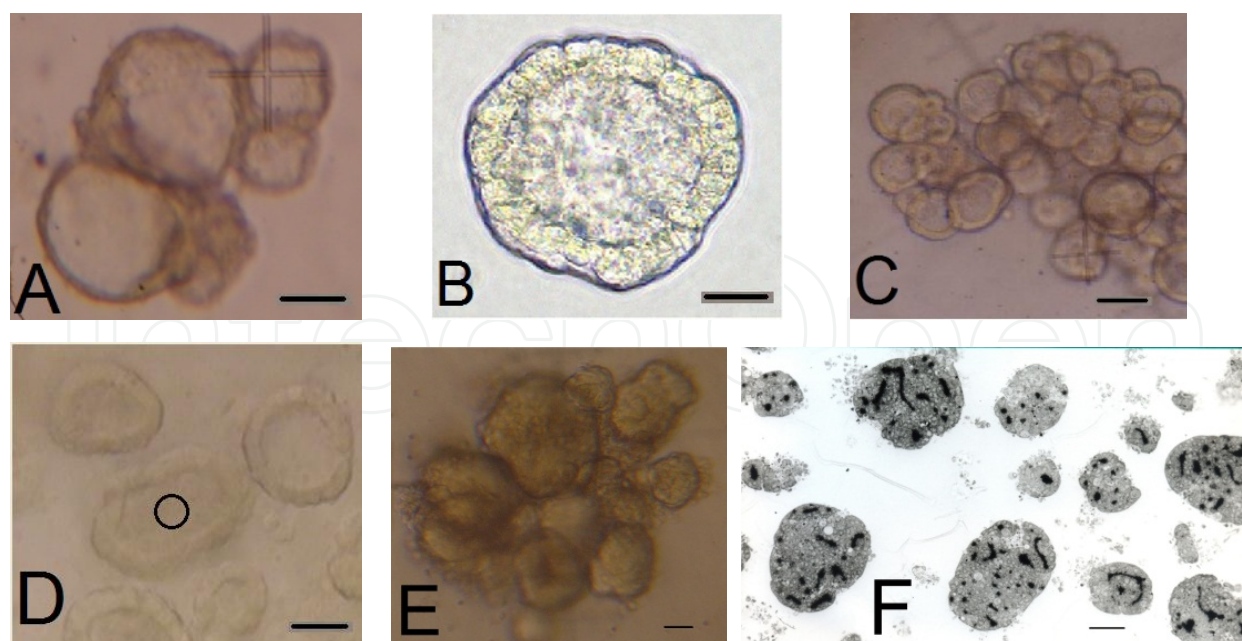
Figure 5. Morphological aspect of follicles in culture. A. Hypothyroid follicle 1 day's culture without TSH; birefringent colloid and thin follicle epithelium can be seen. **B.** Hypothyroid follicle 1 day's culture with 1mU/mL TSH; cubic epithelium and thyrocytes' apical poles typical of a resealed follicle can be seen. **C.** Hypothyroid follicles in 9 days' culture without TSH; the epithelium is cubic and colloid birefringence can be seen in all follicles. **D**. Euthyroid follicle 1 day culture without TSH. A resealed follicle (circle) having irregular contour between thyrocytes and cavity can be seen in the centre. Closed follicles preserve colloid birefringence and regular boundary between thyrocytes and colloid since the start of culture. **E** Pig euthyroid follicles in the presence of 1 mU/mL TSH; follicular cavities are difficult to distinguish. **F.** Autoradiography of rat follicles cultured 12 days in the presence of TSH (1 mU/mL) corresponding to experiment 2, Table 3. Follicle cavities are evident due to the organified iodide found only in very narrow follicular cavities (Scale bar: A and D 50 μm, B 25 μm, C 130 μm, E 100 μm, IM. F 150 μm, OM. Toluidina bleu).

Rat or pig euthyroid follicles without TSH kept the same follicular architecture throughout the whole culture time (Figure 5D). Colloidal cavities became reduced from the third day in the presence of TSH and were difficult to distinguish on day 9 and 12 by IM (Figure 5E); however, they could be seen by autoradiography where only iodide bound to molecules could be identified and they were only located in follicles' very narrow colloidal cavities (Figure 5F). Colloidal cavities' boundaries could also be seen by labelling thyrocytes' apical membrane protein SLC5A8 (short-chain fatty acids transporter) [84].

Rat thyrocyte and pig euthyroid follicle ultra-structure in **one day** culture without TSH (Figure 6A) and with TSH (Figure 6B) was comparable to that for cells *in vivo* (Figure 1C). They conserved their polarity and organelles, but exocytic vesicles were difficult to distinguish from those from endocytosis. RER was more abundant in the presence of TSH and microvellosities were more evident than without TSH.

Thyrocytes had vacuolated and reduced RER and GC following **3 days'** culture without TSH; the GC could be seen in supra-nuclear position (Figure 6C G) as could numerous autophagic vacuoles (Figure 6C arrow) and secondary lysosomes. This became modified in
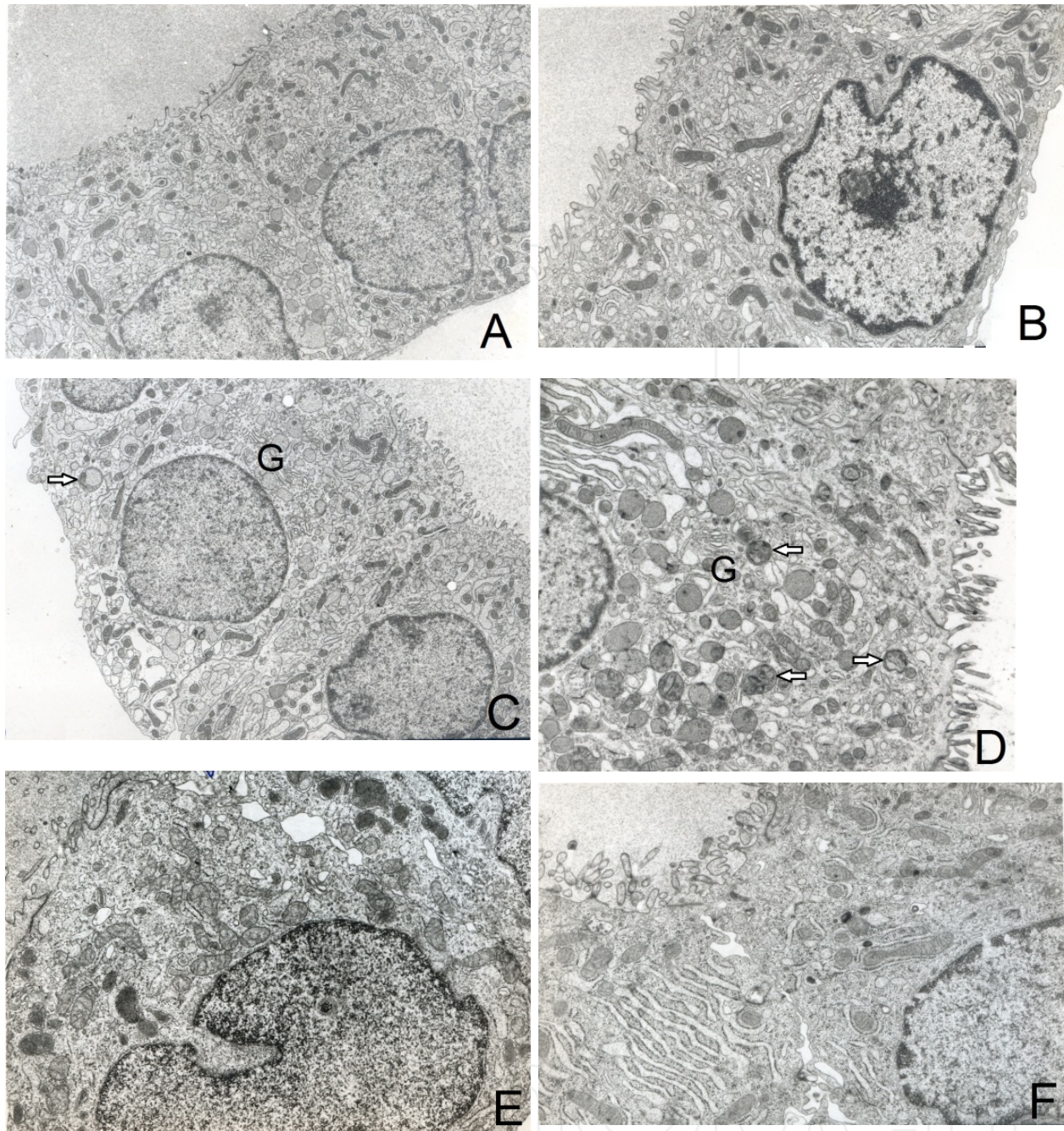
Figure 6. Ultra-structure of thyrocytes from follicles cultured without TSH A 1 day, C 3 days, D 12 days and with 1 mU/mL TSH B 1 day, D 3 days and F 12 days. Binding complexes were preserved in thyrocytes' lateral membrane apical region during all the times with and without TSH. **A.** Thyrocytes conserved their polarisation; microvellosities in contact with electron-dense colloid. Binding complexes were located in lateral membrane's apical region between cells. The rugose endoplasmic reticulum (RER) was found to be slightly vesiculated. **B.** Thyrocyte ultra-structure was comparable to without TSH, even though RER was more abundant. **C. and D.** Ultra-structure was conserved in thyrocytes; the supranuclear Golgi complex (G) and more abundant RER in thyrocytes in the presence of TSH can be seen. Autophagic vacuoles (C. arrow) and secondary lysosomes (D, arrows) with or without TSH can be seen. **E.** Thyrocyte polarity was conserved. Colloid was electron-dense and separated from follicle exterior. Thyrocytes had exiguous RER and G. **F.** Organelles were well conserved. RER was well developed and occupied thyrocytes' apical region (TEM. A and C 7,300 X, B 7,500 X, D 7,438 X, E 9,810 X, F 8,260 X).

the presence of TSH, presenting abundant RER and GC (Figure 6D G) and containing more autophagic vacuoles and secondary lysosomes in thyrocytes' apical region (Figure 6D arrows) than without TSH. RER and GC became more reduced by the **sixth day** without TSH and thyrocytes became thin. Whilst autophagic vacuoles and secondary lysosomes became reduced with TSH, RER and GC also did so by day 3.

Thyrocytes were thin by **day 9** and **12** without TSH and had exiguous RER and GC and reduced microvellosities. The nuclei contained very little heterochromatin (Figure 6E). Follicular centres were very narrow in the presence of TSH, thyrocytes had abundant GC and RER reaching the cells' apical regions (Figure 6F) and the other organelles had the same distribution as on the first days of culture.

Adding 10E-10 M NaI did not modify thyrocytes' follicular architecture or ultra-structure; however, RER and GC were preserved up to day 12 without TSH.

Pig thyrocyte and hypothyroid follicle ultra-structure had exiguous RER and GC when culture began (Figure 7A), like original gland hypothyroids *in vivo*, and culture (even without TSH) developed these organelles from the third day of culture [86]. These hypothyroid follicles' thyrocytes became cubic in the presence of TSH (Figure 5B) from the first day of culture and it was seen that the RER and GC developed and became more evident on the **sixth** and **ninth day** of culture (Figure 7B), similar to rat or pig euthyroid follicle response with TSH, but follicular cavity did not become reduced.

Thyrocytes had normal mitochondria in rat follicle and pig euthyroidic and hypothyroid cultures for all culture times.
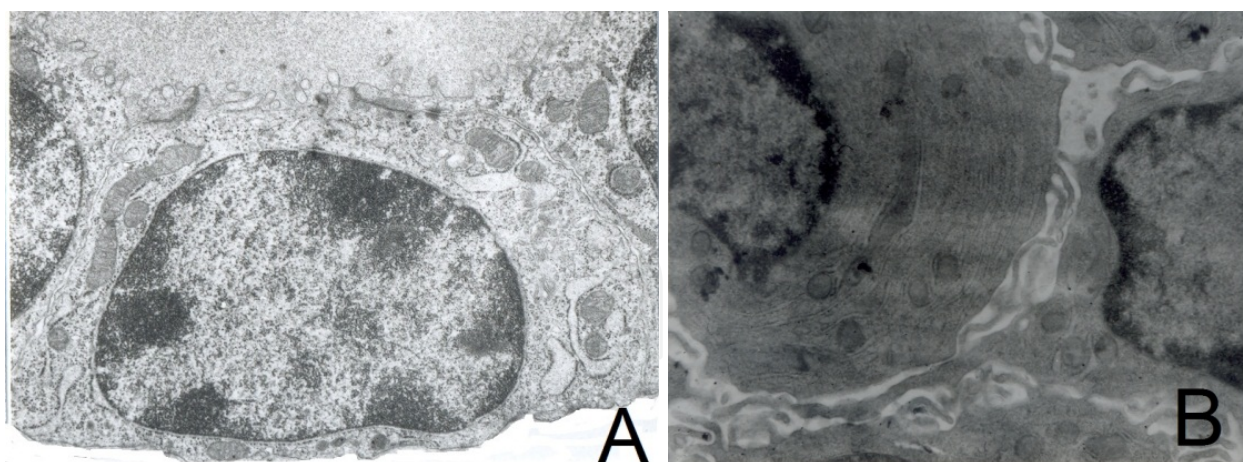

Figure 7. Pig hypothyroid follicle culture. **A.** RER and GC were exiguous and cells were thin 1 day in the absence of TSH. **B.** 9 days in the presence of 1 mU/mL TSH. Cross-section of thyrocyte follicle basal pole. Abundant RER can be seen around the nucleus (TEM. A 7,800 X, B 9,000 X).

Similar culture models to these have been described. Pig follicle culture has highlighted the importance of thyrocyte polarity, but OM morphological study was limited on day 1 [74,94]. Another, dealing with "normal" human follicle culture of thyroidectomy for goitre requiring

TSH [93] had a TEM image showing thyrocytes having the ultra-structure for cells in the process of cell death with lysed mitochondria, without RER or GC. Using this human follicle culture model enabled analyzing the effect of TSH [95] or cytokines [95,96] without presenting culture morphology.

Our results showed that if culture was begun with closed follicles then extracellular matrix support elements were not required [56,57,79,80] nor was TSH for maintaining follicular cavity, as has been described in most pseudofollicle or follicle cultures reconstructed from monolayers, or similar structures called "follicles" [57,50,60,61,62,79,96], thereby demonstrating that if closed follicles are used from the start of culture they conserve their morphology, having the correct polarity as that of their thyrocytes in culture and have a binding complex (tight junction, belt desmosome and spot desmosome) in lateral membranes' apical part as well as *in vivo* [9]. Such closed follicles in culture responded to TSH, like other *in vitro* models [42] or like gland follicles in normal *in vivo* to TSH stimulus [3,25], epithelium thickness, RER and GC becoming increased, and follicular cavities becoming notably reduced [11,83,90]. Follicle response to the absence of TSH both *in vivo* [24] and *in vitro* [42] was also comparable as these organelles became reduced. This effect became reverted *in vivo* when TSH was added, reactivating thyroid functions [11,33,97].

Pig closed and isolated hypothyroid follicles behaved like the gland *in vivo* when the hypothyroid effect was deleted [24], RER and GC increased, follicular epithelium became thin to cubic and culture time became faster in the presence of TSH, but follicular cavity did not become reduced during the 9 days of culture.

Our results thus showed that long-term thyroid follicle function and morphology can be maintained *in vitro,* being equivalent to the gland *in vivo*.

The next section describes the effect of increasing doses of iodide on closed follicles in culture.

5.3. Closed follicle cultures reproduce the Wolff-Chaikoff effect described *in vivo*

5.3.1. Functional study

Follicles were cultured for 1 day before starting to analyse the effect of different iodide concentrations.

Follicles accumulated iodide linearly for 6 h in the presence of 10E-10 and 10E-7 M NaI; accumulation was at its maximum after the first 30 min with 10E-5 M and 10E-3 M NaI (Table 7); they became accumulated 100 times more than with 10E-10 M NaI in the presence of 10E-7 M NaI. Whilst high O/A percentages in follicles were maintained in the presence of 10E-10 and 10E-7 M NaI, organification began after 2 h with 10E-5 M, accounting for only 5% of accumulated iodide, these values being maintained for 6 h, whilst O/A proportion was zero for all times with 10E-3 M NaI (Table 7), even though accumulation could have been 100 times greater to that presented by follicles in the presence of 10E-7 M NaI [98].

	% O/A			
M, NaI	**10E-10**	**10E-7**	**10E-5**	**10E-3**
Culture, hours				
0.5	82	64	0	0
1	84	42	0	0
2	88	90	5	0
3	92	88	3	0
4	95	87	6	0
5	88	89	6	0
6	84	93	4	0

Table 7. Response of organification percentage regarding iodide accumulation (O/A proportion) by rat follicles cultured in the presence of increasing doses of iodide (NaI, M) and Na^{125}I 5 µCi/mL. The follicles were cultured on agarose with 0.5% FCS for 1 day, medium was changed and culture began with NaI and TSH experimental points. Each value was the average of two samples or culture dishes expressed in %/dose/µg DNA.

Iodide accumulation and organification in rat follicles could be assimilated to biochemical reactions after 30 min with and without TSH and thus define a constant (Km) and maximum speed (Vmax). Accumulation apparent Km without TSH was 5x10E-6 M and 10E-7 M de NaI with TSH; iodide organification apparent Km was 5x10E-7 M and was not modified by the presence of TSH (Figure 8). Iodide accumulation by isolated follicles corresponded well with saturable iodide transport characteristics [15,99] and stimulated TSH thereby reducing Km but not Vmax (Figure 8). It should be stated that organification had to be inhibited and
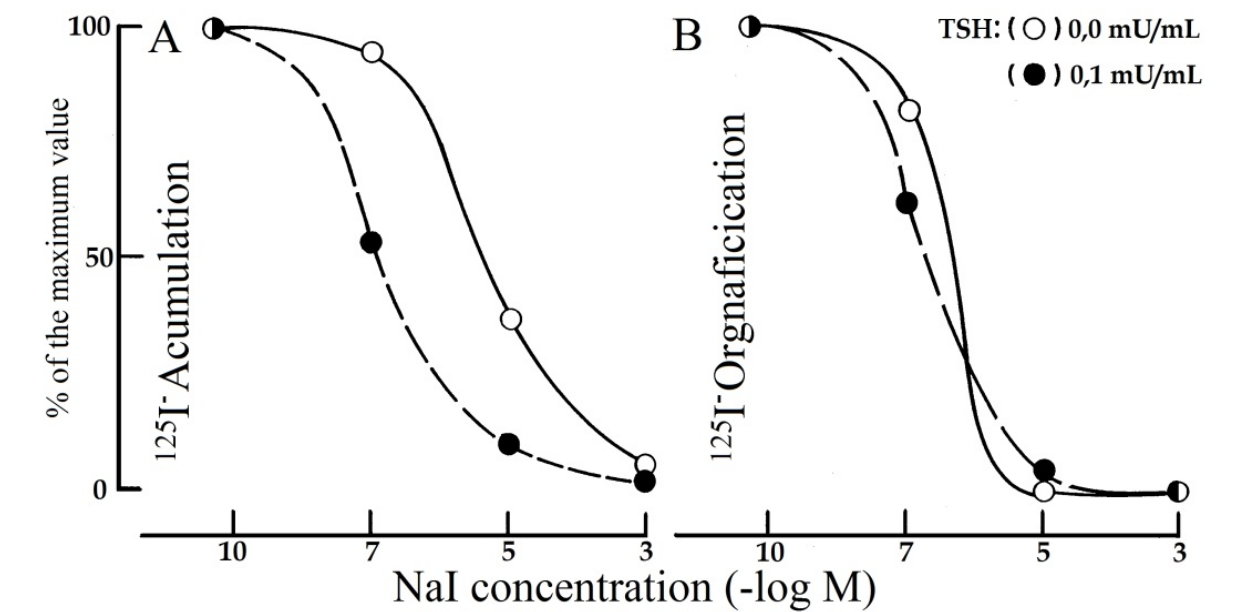

Figure 8. Initial ^{125}I-iodide accumulation (**A**) and organification (**B**) for follicles in culture measured following half an hour in the presence of increasing doses of ^{127}-I Na and 5 µCi/mL ^{125}-I Na expressed in -log M with and without TSH. The follicles were cultured on agarose with 0.5% FCS for 1 day, the medium was changed and culture began with NaI and TSH experimental points. The results were the average of two samples and were expressed as percentage of maximum value.

the time between the end of the culture and the moment when accumulation was determined had to be reduced for measuring exact iodide transport parameters. The washing times meant that part of ^{125}I$^-$ concentrated in follicles would be released before radioactivity was determined and this would induce under-stimulation of accumulation and perhaps overstimulation of O/A percentage.

The presence of TSH for 30 min suppressed the inhibiting effect on iodide organification at 10E-5 M, but did not suppress such iodide effect on organification in the presence of 10E-3 M TSH (Table 8).

	M, NaI	10E-10			10E-7		
Culture	TSH	A	O	% O/A	A	O	% O/A
0.5	-	2,58	1,29	50 ± 5*	360,9	156,34	43 ± 6*
	+	8,06	4,52	56 ± 2*	676,69	406,02	60 ± 14*
3	-	10,88	8,16	75 ± 10*	902,26	525,24	58 ± 7
	+	14,84	11,81	79 ± 1*	1308,27	1079,24	82 ± 1
8	-	35,97	29,84	83 ± 11	2255,64	1602,81	71 ± 4*
	+	32,26	28,06	87 ± 23	1736,84	1371,98	79 ± 10*
12	-	ND			1849,62	1191,13	64 ± 9
	+	ND			1353,38	853,23	63 ± 13
24	-	ND			1714,29	1421,05	83 ± 10
	+	ND			1759,4	1254,17	71 ± 8
48	-	ND			1917,29	1361,98	71 ± 10*
	+	ND			3969,92	3194,23	80 ± 6*
	M, NaI	10E-5			10E-3		
Culture	TSH	A	O	% O/A	A	O	% O/A
0.5	-	14749,09	0	0 *	233469,39	0	0
	+	12480,01	2,63	21 ± 16*	244244,9	0	0
3	-	15272,73	1553,64	10 ± 6	520816,33	0	0
	+	17541,82	2987,27	17 ± 2	466938,78	0	0
8	-	16495,45	2397,15	14 ± 5	574693,88	0	0
	+	20770,91	4450,91	21 ± 2	790204,08	0	0
12	-	ND			* 718367,35	0	0
	+	ND			* 1957551,02	0	0
24	-	ND			* 3071020,41	0	0
	+	ND			* 682448,98	0	0
48	-	ND			664489,8	0	0
	+	ND			484897,96	0	0

Table 8. The effect of I$^-$ dose on the accumulation (A) and organificación (O) of the rat follicles. Follicles cultured on agarose with 0.5% FCS for 1 day; the medium was changed and culture began with different experimental points. The follicles were keep in culture along the time (culture in hours) with 5 μCi/mL Na^{125}I, with (+) or without 0.1 mU/mL TSH (-) and doses of Na^{127}I (NaI). Each value was the average of two samples expressed in μg/dose/μg DNA. This result was representative of 5 independent experiments. O/A: percentage of the proportion O/A ± SD. *p< 0.05. ND: not determinated.

Follicle culture in the presence of the same iodide dose with 0.1 mU/mL and without TSH led up to 8 h with 10E-10 M and 10E-5 M NaI and up to 48 h with 10E-7 M and 10E-3 M NaI. An iodide accumulation function directly proportional to NaI concentration was reproduced as in table 7.

Accumulation in follicles became saturated after 8 h in the presence of **10E-7 M** NaI with and without TSH, becoming slightly reduced at 12 and 24 h and increasing at 48 h; iodide accumulation with TSH was lower at 8, 12 and 24 h than without TSH (significant only at 12 h, $p < 0.05$). Percentage of the proportion O/A was maintained high values with and without TSH, difference with and without TSH at 30 min and 48 h being significant (Table 8).

Iodide accumulation satuproportion was reproduced in follicles in the presence of **10E-5 M** NaI, whether with or without TSH. TSH significantly stimulated organification inhibited by 10E-5 M NaI ($p < 0.05$) at 30 min, even though O/A percentage was slightly higher than at other times (but was not significant) (Table 8).

Iodide accumulation in follicles in the presence of **10E-3 M** NaI with and without TSH reached a *plateau* at 3 h (Table 8) and was greater in follicles without TSH than with TSH at 12, 24 and 48 h; however, this difference was only significant at 12 and 48 h ($p < 0.05$). Iodide accumulation became reduced with and without TSH at 48 h to 3 h values, even though without TSH this was greater but not significantly so. Iodide organification was zero with such strong dose of iodide with and without TSH at all times examined (Table 8).

Pig euthyroidic follicles had the same response as rat follicles when culturing for 48 h in the presence of increasing doses of iodide with or without TSH. Organification was zero at all times in the presence of 10-E3 M NaI with and without TSH (Table 9).

Culture	M, NaI TSH	10E-10 % O/A	N	10E-7 % O/A	N	10E-3 % O/A	N
0.5	-	30.4 ± 0.56	2	30.4 ± 0.63	2	0	3
	+	34.7 ± 9.21	2	72.7 ± 2.84	2	0	3
3	-	67.0 ± 1.54	2	63.2 ± 9.14	2	0	3
	+	79.5 ± 6.79	2	73.6	1	0	3
12	-	71.8 ± 2.35	2	52.4 ± 2.63	1	0	3
	+	78.9 ± 2.63	2	77.6 ± 3.15	2	0	3
24	-	59.7 ± 2.09	2	87.6 ± 1.97	2	0	4
	+	68.8 ± 7.13	2	85.7 ± 0.99	2	0	3

Table 9. The effect of I⁻ dose on the proportion accumulation/organificación pourcentage (% A/O) in euthyroidic pig follicles cultured. Follicles cultured on agarose with 0.5% FCS for 1 day; the medium was changed and culture began with different experimental points. The follicles were keep in culture along the time (culture in hours) with 5 µCi/mL Na^{125}I, with (+) or without 1 mU/mL TSH (-) and doses of Na^{127}I (NaI). Each value was the average of accumulated or organified (O/A) iodide expressed in %/dose/µg DNA per number (N) of culture dishes ± SD.

Pig hypothyroid follicles had a different response to the presence of increasing doses of iodide than rat and pig euthyroidic follicles (Table 10). They accumulated 40 to 100 times

more in the three doses than euthyroidic follicles, presenting organification in the presence of 10E-3 M NaI with or without TSH. Iodide accumulation in these follicles in the presence of perchlorate (30 μM) was only inhibited at 12 h in the presence of 10E-3 M NaI and at 3 h in the presence of 10E-7 M NaI (euthyroidic follicles in the presence of 30 μM perchlorate inhibited iodide capture at all concentrations) and O/A percentages in follicles in the presence of 10E-7 M NaI were greater in the absence of TSH than in their presence (Table 10), such value becoming reduced with culture time.

	NaI	10E-10 M		10E-7 M		10E-3 M	
Culture	TSH	% O/A	N	% O/A	N	% O/A	N
0.5	-	77.9 ± 0.61	4	74.9 ± 0.63	4	6.3 ± 0.63	3
	+	57.3 ± 0.78	4	71.6 ± 2.84	4	15.1 ± 0.63	3
3	-	75.8 ± 0.49	4	68.7 ± 9.14	4	5.4 ± 0.63	3
	+	60.6 ± 0.68	4	57.2 ± 9.14	4	3.0 ± 0.63	3
12	-	ND		87.3 ± 2.63	4	10.4 ± 0.63	3
	+	ND		72.8 ± 3.15	4	3.8 ± 0.63	3
24	-	ND		44.3 ± 1.97	4	11.6 ± 0.63	4
	+	ND		28.7 ± 0.99	4	9.7 ± 0.63	3

Table 10. The effect of I- dose on the proportion accumulation/organificación pourcentage (% A/O) in pig hypothyroid follicles cultured. Follicles cultured on agarose with 0.5% FCS for 1 day; the medium was changed and culture began with different experimental points. The follicles were keep in culture along the time (culture in hours) with 5 μCi/mL Na^{125}I, with (+) or without 1 mU/mL TSH (-) and doses of Na^{127}I (NaI). Each value was the average of iodide accumulated or organified (O/A) iodide expressed in %/dose/μg DNA per number (N) of culture dishes ± SD. ND: not determinated.

Iodide accumulation values in the presence of 10E-3 M NaI in hypothyroid follicles were 2 times greater in follicles in the presence of TSH than without TSH, had organification in similar proportions to euthyroidic follicles in the presence of 10E-5 M NaI. Percentage of the proporction O/A was higher at 30 min without TSH than with TSH; it was lower at the other times. Without TSH increased with culture time whilst values became reduced regarding time with TSH (Table 10). Organification in this case was not nil, but the organified iodine did not exceed 11% for accumulated iodide.

Mouse follicles cultured 3 days in the presence of 10E-7 M NaI was more intense in the presence of reactive species at the boundary between apical membrane microvellosities and colloid and the oxide reduction system became completely closed down with an excess of iodide (10E-4 M NaI) [100]. This proved that an excess of iodide inhibited the enzymes responsible for organification in closed follicles as we have thought should be in the gland *in vivo.*

Euthyroidic follicles accumulated iodide regarding medium constant proportion concentration. Accumulated iodide was organified in the presence of 10E-10 M and 10E-7 M NaI; organification was extremely reduced in the presence of 10E-5 M and zero with 10E-3 M NaI as described *in vivo* [1]. Accumulation was greater in follicles without TSH than with TSH in the presence of 10E-3 M NaI at 12, 24 and 48 h; this reduction was similar to that of the gland *in vivo* [101,102].

Hypothyroid follicles in the presence of 10-3 M NaI accumulated more iodide without TSH than with TSH at the beginning (30 min), even though proportion of O/A percentage was greater at 30 min than at other times; this effect (different to euthyroidic ones) was similar to that described *in vivo* in hypothyroid glands [28]. They also responded by reproducing the effect observed in animals suffering experimental goitre as, when excess iodide was added to food, they did not respond to TSH [24]. 50% of human endemic goitre has responded to an iodine-rich diet and some of these to TSH [25] as hypothyroid follicles in the presence of TSH and 10E-3 M NaI have responded by becoming less organified than in the absence of TSH. It should be stated that there is no consensus regarding what should be the correct dose for defining low, medium or high iodide concentration in human alimentation [25].

Closed and isolated follicles thus responded to 10E-5 M and 10E-3 M NaI inhibiting iodide organification in a similar way to that described for the gland *in vivo* in 1948 [1] (i.e. reproducing the Wolff-Chaikoff effect).

5.3.2. Morphological study

Hypothyroid follicles conserved follicular architecture during 48 h of culture in the presence of different doses of iodide with and without TSH (Figures 9A, 9B and 9C). Rat follicles and pig euthyroidic follicles at all iodide doses in the presence or absence of TSH conserved follicular architecture for 48 h (Figure 9D).

Figure 9. Appearance of pig follicles cultured on agarose with 0.5% FCS for 1 day; the medium was changed and culture lasted 48 h. A. Hypothyroid s follicles in the presence of 10E-10M NaI. **B.** Hypothyroid follicles in the presence of 10E-7M NaI. **C**. Hypothyroid follicles in the presence of 10E-3M NaI. **D.** Euthyroidic follicles in the presence of 10E-3M NaI. The follicular architecture of the gland *in vivo* was conserved in all cultures; hypothyroids (A, B and C) had thin epithelium and euthyroidic (D) ones cubic epithelium (IM. Scale bar: A, B, C 800 μm, D 200 μm).

The Trypan blue exclusion exam of thyrocytes from follicles cultured with 10E-3 M NaI did not have an alteration to their membranes and excluded the stain, whether being pig euthyroidic follicles cultured 48 h (Figure 10A) or rat ones cultured for 6 days (Figure 10B). The cells of cell aggregates which did not have follicular structure became stained (Figure 1C), the same as isolated cells (Figure 10B) or those found in follicles.

The ultra-structure of thyrocytes in all treatments and times preserved cell polarisation and organelle distribution (Figure 11), like the gland *in vivo* (Figure 1C); endocytic vesicles can be seen in thyrocytes' apical region (Figure 11) like follicles after 3 days of culture (Figures 6C and 6D).
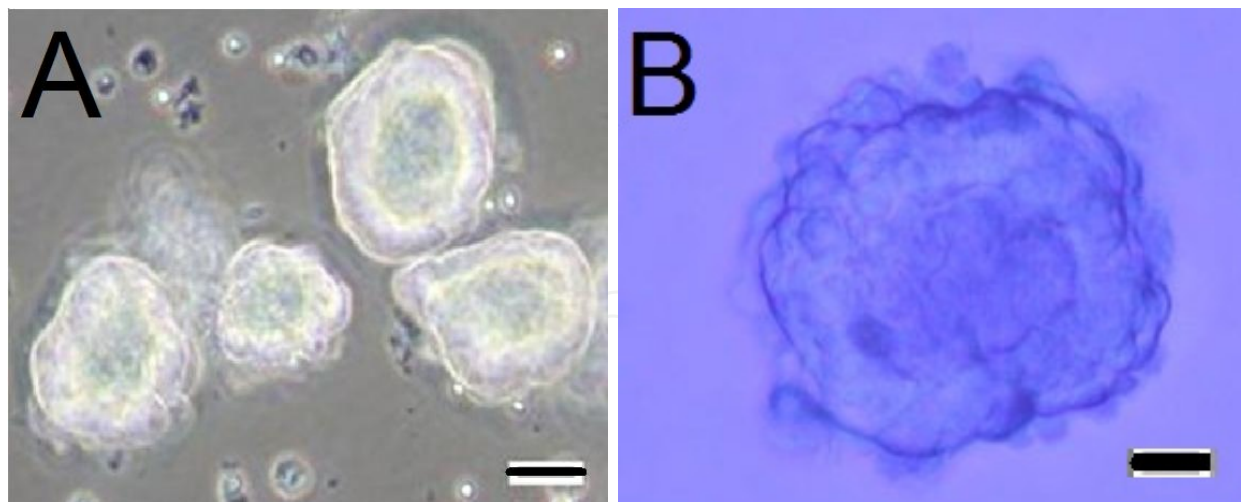
Figure 10. Trypan blue exclusion exam of culture aliquots for follicles cultured on agarose with 0.5% FCS 1 day; the medium was changed and culturing involved 10E-3 M NaI. **A.** 2 day rat follicle culture. **B.** 6 day pig euthyroid follicle culture. The thyrocytes from the follicles excluded the stain whilst isolated cells did not exclude it, whether separated from the follicle (A) or on the follicle (B) (IM, Scale bar A 60 μm, B 15 μm).
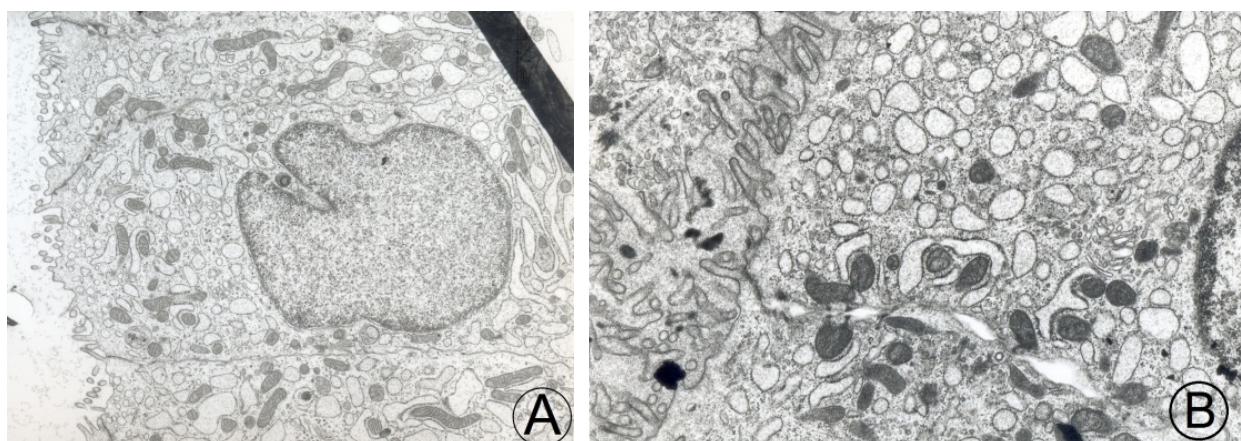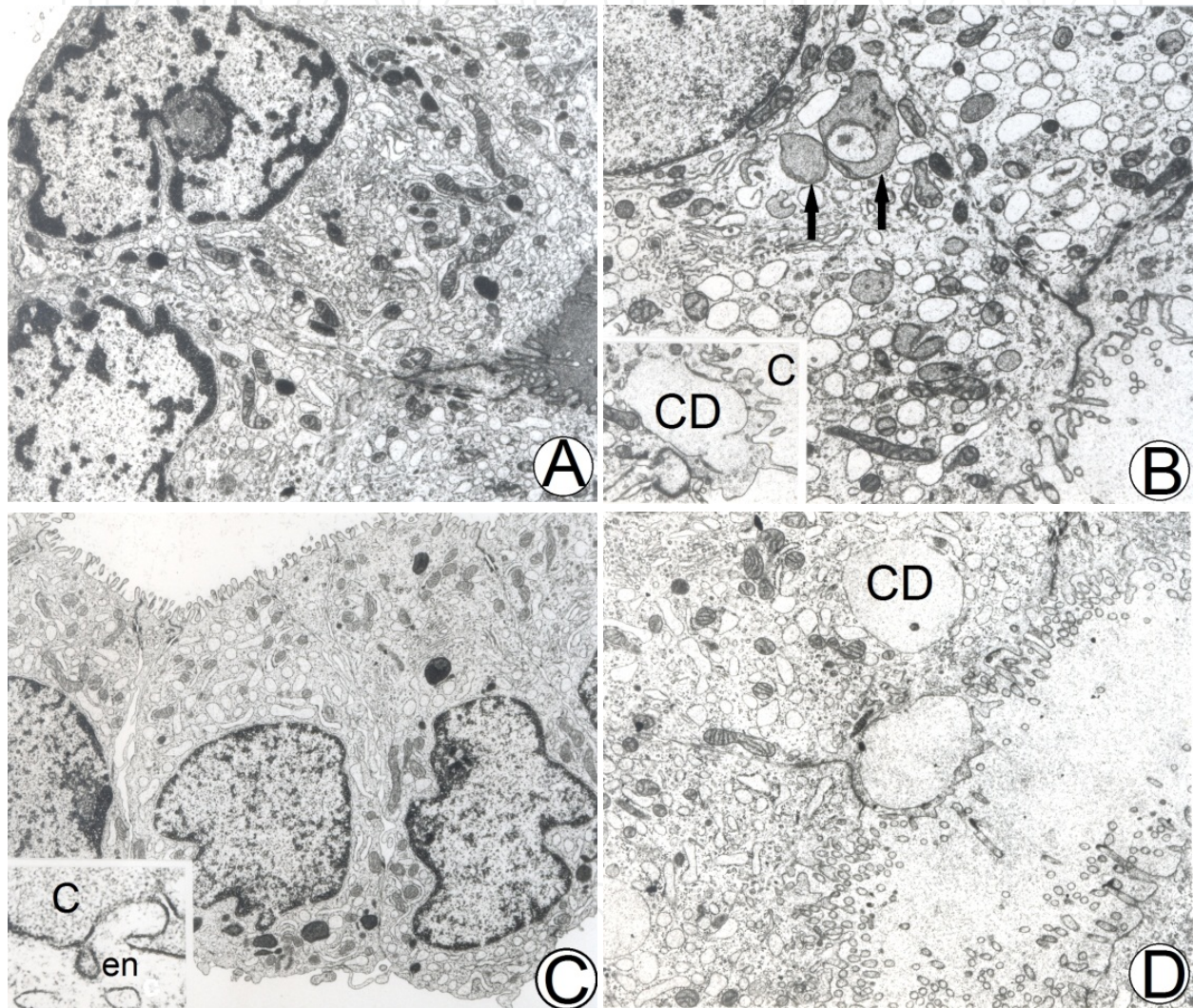

Figure 11. The ultra-structure of rat follicle rat thyrocytes cultured on agarose with 0.5% FCS for 1 day; the medium was changed and 48-h culture involved different experimental points: **A.** 10E-7 M NaI. **B.** 10E-3 M NaI + 0.1 mU/mL TSH. Thyrocytes kept their polarisation; microvellosities were in contact with electron-dense colloid. The binding complexes were located in the lateral membrane's apical region between cells. The RER was slightly vesiculated. Thyrocytes did not have cytological differences regarding iodide dose; follicular centres only became narrowed in the presence of TSH (B) (TEM. A 8,720 X, B 10,720 X)

Follicles in the presence of TSH narrowed their follicle centres at all iodide doses used (Figures 12B and 12D), but did not undergo any ultra-structural modification in strong iodide concentrations: 10E-5M or 10E-3 M NaI. Organelle distribution was comparable to normal gland *in vivo* (Figure 1C) or follicles in long-term culture (Figures 6C and 6D).

Lysosome fusion occurred in follicles in the presence of 10E-10 M NaI and 0.1 mU/mL TSH for 8 h (Figure 12B) while this did not happen in thyrocytes' apical region (Figure 12B insert)

in the absence of TSH (Figures 12A and 12C). This was also observed en thyrocytes from follicles cultured for 8 h with 10E-3 M NaI and TSH, but colloid droplets were only located in the apical region in this dose (Figure 12D). Thyrocytes also had endocytic vesicles in this dose without TSH but also located in cells' apical region (Figure 12C). Thyrocytes ultra-structure in the presence of strong concentrations of iodide did not have morphological modifications, or distribution of organelles regarding normal or non-stimulated cells *in vivo* (Figure 1C) or *in vitro* (Figures 6C and 6D).

Figure 12. The ultra-structure of rat follicle thyrocytes cultured on agarose with 0.5% FCS for 1 day; the medium was changed and 8 h of culture involved different experimental points: A. 10-E10 M NaI. B. 10-E10 M NaI + 0.1 mU/mL TSH. C. 10-E3 M NaI. D. 10-E3 M NaI + 0.1 mU/mL TSH. A. The ultra-structure of thyrocytes in the absence of TSH was identical to that of thyrocytes from follicles cultured for 1 day. B. Lysosomes close to the nucleus and few *colloid droplets* located in the apical pole (insert) were observed in the presence of TSH. C. They were well conserved in the presence of TSH, even in this strong dose of NaI. Endocytic vesicles were present in such strong dose of iodide. D. Colloid droplets were also observed in the presence of TSH, having the same density as colloid and were located in the thyrocytes' apical pole. Follicle centre was narrow and had abundant microvellosities (TEM. A 8,720 X, B 12,510 X, box 10,720 X, C 7,430 X, box 70,950 X, D 13,450 X).

The thyrocytes from follicles cultured from 30 min up to 6 days in the presence of 10E-3 M NaI did not have cytotoxic signs and all follicular cells were viable, like normal animals' thyroid glands' *in vivo* when fed with excess iodide for 3 weeks [103].

Open human follicle culture in the presence of 10E-3 M NaI with and without TSH for 24 h had ultra-structural alterations related to cytotoxicity [92] involving free radical attack and lipid peroxidation. Excess iodide in pig follicles led to thyrocyte apoptosis because iodine production in lactone became reduced, but did not present morphology. Our results showed that conserving closed follicles did not lead to signs of cell death with 10E-5 M or 10E-3 M NaI or disorganisation or alteration of thyrocyte ultra-structure. The difference with [92] and [94] was that they were open follicle cultures and thyrocytes died simply because they were cultured on a support which inhibited cellular adhesion, like our cell aggregates.

Closed follicles were present (Figure 12), as described for the gland. There was apical membrane turnover between microvellosities which is important for maintaining Tg synthesis and its secretion to colloid [8]. Coated endocytic vesicles (Figure 12C insert) were also present in the base of microvellosities, like micro-endocytosis *in vivo* [5], and those stimulated by TSH formed pseudopods and colloid droplets (Figures 12B insert and 12C letter DC), called *in vivo* macro-endocytosis [10]. Thyrocyte fusion with prelysosomes or late endosomes from the lysosome route was also observed (Figure 12B arrows) for Tg degradation and thyroid hormones were formed *in vivo* [19] and *in vitro* [18].

Closed rat and pig euthyroidic follicles responded to increasing doses of iodide, as *in vivo*, thereby producing the Wolff-Chaikoff effect [1], and presented no modifications in thyrocytes' follicular architecture or ultra-structure, being comparable to a gland *in vivo*.

5.3.2.1. Na^+/I^- symporter determination

Many thyrocyte culture studies have described reduced RNAm and NIS protein expression when maintained in the presence of strong iodide concentrations (10E-6 to 10E-4 M of iodide). We wanted to determine NIS in rat follicles cultured with strong iodide concentrations with and without TSH 0.1 mU/mL.

Follicles cultured at **12 h** in the presence of 10E-7 M NaI and without TSH had NIS in basolateral membranes (Figure 13A) and labelling was more intense in lateral membranes in the presence of TSH (Figure 13B). In the presence of 10E-3 M NaI NIS was mainly located in vesicles between nucleus and basolateral membranes (Figure 13C), and in the presence of TSH; as well as being presented in vesicles they were observed in basolateral membranes (Figure 13D). It could have been that inhibiting vesicular movement in the presence of 10E-3 M NaI in the apical region (Figures 11C and 11D), as well as inhibiting the movement of the vesicles forming in the basement region with NIS symporter for avoiding excessive iodide entry to thyrocytes.

The NIS symporter was located in the basolateral members in the presence of 10E-10 M NaI at 48 h (E and F), labelling being more intense in the presence of TSH. The NIS symporter was found in the cytoplasmatic vesicles with TSH (F). NIS symporter expression in the presence of 10E-3 M NaI for 48 h (G and H) was so low that confocal microscope parameters
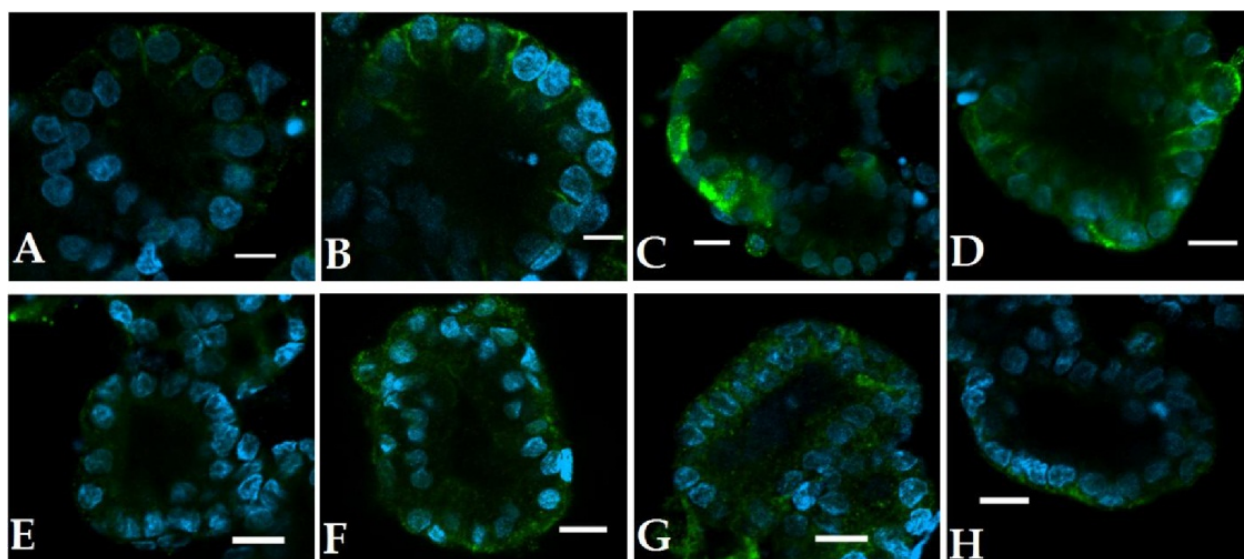
Figure 13. Indirect immunofluorescence of NIS symporter (green) expression and localisation and DAPI-labelled nuclei (blue) in follicles isolated from rats cultured for 1 day on agarose with 0.5% FCS for 1 day; the medium was changed and were cultured for 12 h (A, B, C and D) and for 48 h (E, F, G and H), with different experimental points: NaI with and without 0.1 mU/mL TSH. **A.** 10E-7 M NaI, **B.** 10E-7 M NaI + TSH. **C.** 10E-3 M NaI. **D.** 10E-3 M NaI + TSH. **E.** 10E-7 M NaI. **F.** 10E-7 M NaI + TSH. **G.** 10E-3 M NaI. **H.** 10E-3 M NaI + TSH. NIS symporter was located in the basolateral membranes in the presence of 10E-10 M NaI at 12 h (A and B). TSH intensified labelling (B). NIS symporter was located in cytoplasmatic vesicles near thyrocytes' basolateral membranes in the presence of 10E-3 M NaI at 12 h (C and D). There was more intense labelling in some thyrocytes' basolateral membranes in the presence of TSH (D). [104]

(laser intensity, detector gain, scanning time) had to be adjusted again to increase labelling intensity. NIS protein was mainly observed in cytoplasmatic vesicles in these follicles, being more intense than labelling without TSH (G). Basement membranes had exiguous NIS labelling (CM. Scale bar: 10 μm).

The NIS symporter in follicles cultured for **48 h** in the presence of 10E-7 M NaI were located in vesicles and basolateral membranes (Figure 13E); TSH intensified such labelling (Figure 13F). NIS expression was very reduced regarding the other treatments in the presence of 10E-3 M NaI with and without TSH and microscope parameters had to be readjusted for observing fluorescence. NIS was located in cytoplasmatic vesicles in this strong dose of NaI and without TSH (Figure 13G). TSH was located in vesicles in the base region but labelling was less intense (Figure 13H) than with TSH.

NIS has normally been located in thyrocytes' basolateral members *in vivo* [13], and a reduction in its normal expression has been associated with escape from the Wolff-Chaikoff effect [30] following 48 h in the presence of strong iodide concentrations [29]. Being found in vesicles has reduced NIS in its normal position for thyrocytes from follicles in the presence of a strong dose of NaI [104] and has thus suppressed I transport for thyroid hormone production.

These results were similar in the FRTL5 cell line where the same dose did not alter NIS RNAm percentage, but protein became reduced by 50% and 78 % at 24 and 48 h, respectively [32]. NIS RNAm became reduced in dogs with goitre at 48 h with a comparable

iodide dose [105]. This was perhaps presented by reduced AM2Pc levels at 48 h, as excess I⁻ inhibited an increase in AMPc stimulated by TSH in hypophysectomised rats [106] and mice [34], which could have explained the low NIS level during Wolff-Chaikoff effect and in follicles at 48 h in the presence of excess iodide (Figure s13G and 13H). Excess I⁻ inhibited IP3 production and increased Ca^{2+} flow induced by TSH, which could have led to reduced peroxide production during Wolff-Chaikoff effect [107,108]. The organification observed in the presence of 10E-5 M (Table 8) did not completely inhibit TPO and had no effect on NIS in the presence of 10E-3 M NaI (Figures 13E and 13H), thereby demonstrating that organification depends on TPO and not on NIS as in cells transfected with the TPO gene [34]. TSH did not stimulate the organification of iodide captured in 10E-3 M but did so in 10E-5 M NaI (Tables 8 and 10), as it has been described that the effect of TSH on thyroid physiology becomes reduced in the presence of excess I⁻, meaning that antagonic roles are assumed *in vivo* [102].

TSH modulated relative NIS expression and its subcellular localisation in the thyrocytes of isolated and closed follicles *in vitro*. These results were similar to those found in FRTL5cells, where it has been demonstrated that *de novo* synthesis [32], half-life time, NIS targeting and/or retention regarding cytoplasmatic membrane requires TSH to be located throughout cell membrane, due to loss of polarity [109].

Thyrocyte disposition in follicles has not been necessary for iodide accumulation, since it has been present in foetal thyroids before follicular lumen formation [110] and also in primary cultures from normal thyrocytes [111] or goitre patients [112] and in the FRTL cell line [113]; however, these cultures have required TSH, hormones and other molecules for maintaining them. Nevertheless, isolating the colloidal cavity from the exterior must be ensured for iodide accumulation and incorporation in Tg, T3 and T4 hormone synthesis, as demonstrated with rat or pig isolated and closed follicle cultures.

Rat and pig follicles thus inhibited iodide organification in the presence of strong concentrations of iodide, i.e. performed the Wolff-Chaikoff effect. Neither thyrocytes' follicular architecture nor ultra-structure was modified and no sign of cell death was presented. The TSH and iodide effects observed *in vivo* during the Wolff-Chaikoff effect were reproduced.

6. Conclusion

Loss of follicular structure during the first 24 h of culture has been the main drawback of *in vitro* thyroid studies and, therefore, hormone synthesis. It is not enough to conserve thyrocytes' apical-basal polarity in culture in this specific tissue for maintaining colloid's extracellular functions for the enzymes implicated in iodide fixation on Tg and hormone synthesis.

We have shown that follicular architecture must be conserved in culture, especially the follicular cavity isolated from extracellular medium as this is indispensable for maintaining ultra-structure and the polarity of thyrocytes around the follicular cavity; such premise

conserving the idea of Tg19S synthesis usually being glycosylated and iodised, as also T_3 and T_4 hormone synthesis. Follicular morphological conservation is necessary for reproducing the Wolff-Chaikoff effect *in vitro*, as has been described *in vivo*. NIS symporter localisation in thyrocytes depends on I and TSH concentration.

This culture may be used for obtaining follicles from pathologies of human tissue whose epithelium may be thin plate-like cells for *in vitro* studies in controlled and homologous conditions regarding the pathology *in vivo*. It will also enable studying normal or pathological thyroid's physiological, cellular and molecular mechanisms (for example CLC-5 channel) in a homologous model of the gland *in vivo*.

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