Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 3, 413-419

An updated investigation on the antenatal development of the thyroid gland in white New Zealand rabbit with morphometric analysis

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ARTICLE INFO

Recieved: 21 October 2023

Accepted: 11 February 2024

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Keywords:

Embryo
Primitive pharynx
Parafollicular cells
Rabbit
Thyroid primordium
Thyroid follicles

ABSTRACT

The prenatal development of the thyroid gland was studied on 30 rabbit embryos and fetuses of both sexes. Their ages ranged between 9 days old till the day of birth. The thyroid anlage appeared as an endodermal thickening in the floor of the primitive pharynx on the 9th day of gestation. On 11 days old embryos, the thyroid bud was connected to the pharyngeal endoderm by the thyroglossal duct which began to disintegrate on the 12th day of gestation. The bilobation of primitive thyroid gland was exhibited with the beginning of isthmus organization on the 16th days of gestation. The first evidence of follicular organization appeared at the 20th days of gestation with many small follicles disseminated within the gland. The first morphological sign of functional differentiation of the thyroid gland appeared on the 22nd days of gestation as PAS positive thick rim of pre colloid material in apical parts of the follicular cells in some follicles. Very few parafollicular cells began to scatter among follicular cells at the 22nd days old fetuses. The definitive thyroid follicles storing vacuolated typical colloid organized at the 28th days of gestation and stained somewhat strongly with Eosin and PAS.

Introduction

The rabbit is the best experimental animal of choice to be used in embryological research, as it is spontaneously ovulated animal. This allowed accurate pregnancy timing, in addition to the number and size of its fetuses are large and easier to be examined than the other rodents (Gibson *et al*., 1996; Haddad *et al*., 2021).

The thyroid gland is one of the most important endocrine glands. It consists of two types of cells: Follicular cells and Parafollicular (C-cells). Follicular cells are responsible for the production of thyroid hormones including, Thyroxine (T4) and Triiodothyronine (T3) which regulate the metabolic activities of all body cells. Also, they are required for normal vertebrate development. Parafollicular cells secrete Calcitonin hormone which contributes to serum calcium homeostasis by regulating serum calcium level (De Felice and Di Lauro, 2004; Rohr, 2007; Kameda *et al*., 2009; Haschek *et al*., 2010). The rabbit thyroid gland is surrounded by thin capsule of collagen fibers. Its parenchyma subdivided into lobules composed of variable sized follicles (Nasser, 2014).

The thyroid gland is the first of the body's endocrine glands to develop. It has a double embryonic origin including the endodermal cells of the primitive pharynx, and the ectodermal neural crest cells (Ultimobranchial bodies). The thyroid diverticulum develops as a ventral midline endodermal thickening in the floor of the developing pharynx at a level in between the first and second pharyngeal arches at foramen cecum. Initially, the thyroid primordium is attached to the foregut by the thyroglossal duct which later is obliterated. Then, it extends caudally losing its connection with the foregut. After that it becomes ventral to the developing trachea. The blind end of the primordium forms two lobes connected by an isthmus of glandular tissue (Noden and De Lahunta, 1985; McGeady *et al*., 2017; Kumar *et al*., 2018 and Arrangoiz *et al*., 2018).

The thyroid gland is formed by fusion of three anlagen that develop from the anterior foregut. The median anlage (thyroid diverticulum) which gives rise to follicular cells is merged with a pair of lateral anlagen termed as the ultimobranchial bodies. The latter deliver the parafollicular C-cells to the developing thyroid gland (Rohr, 2007; Fagman and Nilsson, 2010; Gevers *et al*., 2016). The thyroid mass is formed of solid cords of cells that arrange into small cellular clusters with a narrow lumen. Consequently, cells arrange in a single layer around a lumen forming the typical thyroid follicles (Khatawkar and Awati, 2015; Pradhan *et al*., 2021).

Morphological abnormalities and dyshormonogenesis may be caused by deviations in any of the critical stages of thyroid development or thyroid hormone synthesis (Ordookhani *et al*., 2007). The recent studies concerning the prenatal development of the thyroid gland in rabbit were meager. So, the present work was carried out to elucidate some investigations on its differentiation in New Zealand rabbit as a trial to play a part in the improvement of the treatment of the thyroid gland disorders.

Materials and methods

The present work was carried out on 30 rabbit embryos and fetuses of both sexes. Their ages ranged between 9th days old till the day of birth (30th days old). The specimens were obtained from normal, apparently healthy white New Zealand pregnant does. They were collected from the Unit of Experimental animals, at the Faculty of Veterinary Medicine and Faculty of Agriculture Farms, Zagazig University. The pregnant does were anesthetized with a mixture of 35 mg/kg ketamine Hcl (KETALAR, 100 mg/ml, Pfizer, NY) and 5 mg/kg xylazine (Xylaject, 20 mg/ml, ADWIA, Egypt) injected intramuscularly according to Enoka (2013). The pregnant

ISSN: 2090-6277/2090-6269/

does were sacrificed and the embryos and fetuses were taken from the uterus shortly after evisceration and freed from their fetal membranes. For the histological study, the entire embryos of the early stages from the 9th to 20th day of gestation were taken intact. While, in the older stages from the 22nd day to the day of birth the neck region containing the thyroid gland is obtained.

The specimens were fixed either in Bouin's solution for 4-8 hours followed by 70% Ethyl alcohol or in Neutral Buffered Formalin 10% for 24-48 hours. Then, they were subjected to dehydration, clearing, embedding, cutting, and staining with different stains including Hematoxylin and Eosin, Periodic Acid Schiff (PAS), Masson's Trichrome stain, and Gomori's Silver Impregnation stain according to Drury and Wallington (1980) and Suvarna *et al.* (2019). All stained sections were examined with a standard light microscope (Objectives 4, 10, 40) and photographed with a digital camera (Leica, DM500, ICC50W, Germany).

For semithin sections, specimens were fixed in 2.5% Glutaraldehyde in 0.1 M Sodium Cacodylate Buffer and post-fixed in 1% Osmium Tetroxide buffered to pH 7.4 for 2 hours at 4°C. Afterwards the specimens were dehydrated in increasing series of Ethyl Alcohol and embedded in Epoxy Resin. Then, sections (0.5 µm in thickness) were stained with Toluidine Blue according to Chiu *et al.* (1993).

Morphometric measurements were performed by using Leica Q500 imaging analysis system in the Department of Anatomy and Embryology at Faculty of Medicine, Zagazig University. The following parameters were measured; the long diameters of small, medium, and large sized follicles (µm) of 20 days old fetuses till the day of birth. Also, the area percentage of mesenchymal interstitial connective tissue of 14 days old embryos till the day of birth. All parameter measurements were detected in several selected stained sections at 40X magnification.

Statistical analysis

All data were expressed in the Department of Statistics at Faculty of Veterinary Medicine, Zagazig University. The values were presented as Means ± standard Error of Mean (SEM). The data were subjected to the Statistical Package for Social Sciences (SPSS-16.; Chicago, IL, USA) software and one-way Analysis of Variance (ANOVA) at 95 % level of confidence. Significant differences among the means were determined by Tukey's Kramer HD Test considering $P < 0.05$ as significant; No.=10 per each studied age (Brooks and Johanson, 2011).

Results

In rabbit embryos on 9 days old: The thyroid anlage appeared as an endodermal thickening in the floor of the primitive pharynx cranial to the dorsal aorta. This median thickening deepened forming a shallow evagination of the pharyngeal endoderm, at the junction in between the first and second branchial arches (Fig. 1A).

In rabbit embryos on 10-12 days old: The thyroid anlage became more crowded with endodermal cells forming the thyroid bud. The latter appeared as a pseudostratified endodermal outpouching penetrating the underlying mesenchymal tissue. The thyroid bud was connected to the pharyngeal endoderm by a thin cord of undifferentiated endodermal cells termed the thyroglossal duct. The thyroid bud was located cranial to the dorsal aorta and separated from its wall by a thin rim of mesenchymal cells. The thyroid progenitor cells were formed from irregularly arranged undifferentiated endodermal cells with indistinct cell boundaries. They appeared with darkly stained round to oval nuclei and scanty cytoplasm (Fig. 1B and 1C).

At the end of this stage, the thyroid bud descended caudally with gradual degeneration of the thyroglossal duct. Subsequently, it incompletely detached from the endoderm of the primitive pharynx. The proliferation of thyroid bud increased and became more elongated with its caudal end tapered off toward the apical portion of the dorsal aorta (Fig. 1D).

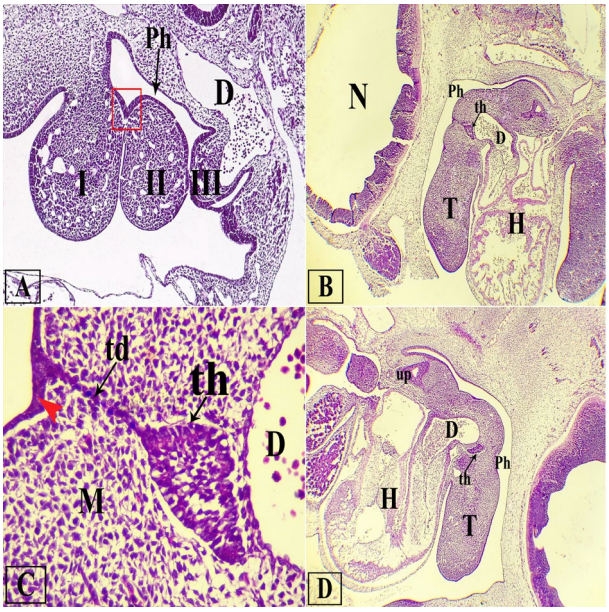

Fig. 1. Photomicrographs of (A) longitudinal section of 9th days old rabbit embryo H&E stain, 10X; (B) longitudinal section of 11 days old rabbit embryo H&E stain, 4X; (C) High magnification to (B) H&E stain, 40X and (D) longitudinal section of 12 days old rabbit embryo H&E stain, 4X showing; thyroid anlage (red square); primitive pharynx (Ph); first, second and third branchial arches (I, II, III), dorsal aorta (D); thyroid bud (th); neural tube (N); primitive tongue (T); Primitive heart (H); mesenchymal tissue (M); pharyngeal endoderm (red arrowhead); thyroglossal duct (td); ultimobranchial-parathyroid IV complex (up).

In rabbit embryos on 13 days old: The thyroid bud was completely separated from the pharyngeal endoderm. The developing thyroid gland had migrated further caudally from the pharyngeal floor to reset as a cap like structure around the primitive common carotid artery. The thyroid bud consisted of solid mass of endodermal cells that surrounded by a highly vascular undifferentiated mesenchymal tissue. The epithelial cells of this solid mass had round to oval vesicular nuclei with little chromatin material and distinct nucleoli (Fig. 2A and 2B).

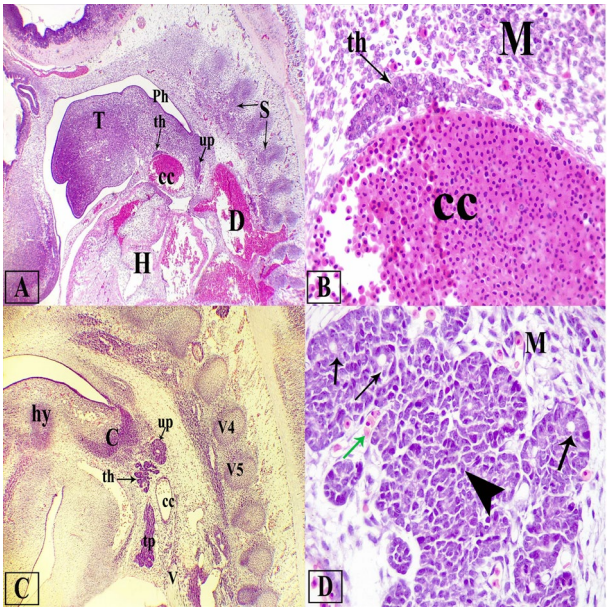

Fig. 2. Photomicrographs of (A) longitudinal section of 13t days old rabbit embryo H&E stain, 4X; (B) high magnification to (A) H&E stain, 40X; (C) longitudinal section of 14th days old rabbit embryo H&E stain, 4X and (D) high magnification to (C) H&E stain, 40X showing, thyroid primordium (th); pharyngeal floor (Ph); the primitive common carotid artery (cc); Dorsal aorta (D), primitive heart (H), somite differentiation (S), primitive tongue (T), ultimobranchial-parathyroid IV complex (up), a highly vascular undifferentiated mesenchymal tissue (M); primitive hyoid bone (hy); laryngeal cartilage (C), the fourth (V4) and fifth (V5) primitive cervical vertebrae; thymus-parathyroid III complex (tp), primitive vagosympathetic nerve trunk (V); the primitive thyroid mass consisted of cell conglomerates (black arrowhead) at the center of the gland, few radially arranged cell cords with intercellular space (black arrows) at the periphery; primitive blood capillaries (green arrow) invading the periphery of the primordium.

In rabbit embryos on 14 days old: The primitive thyroid gland migrated further caudally to the primitive hyoid bone and laryngeal cartilages within the cervical undifferentiated mesenchyme. It extended till the level in between the fourth and fifth primitive cervical vertebrae. The developing thyroid gland was related dorsolaterally to the Ultimobranchial-Parathyroid IV complex and ventrolaterally to the developing Thymus-Parathyroid III complex (Fig. 2C). The proliferating primitive thyroid gland consisted of irregularly and compactly arranged cell conglomerates generally at the center of the gland. At the periphery of the primordium the solid cell clusters were broken into anastomosing solid cell cords through ingrowth of the surrounding vascular mesenchymal elements. A small number of epithelial cells in the cell cords at the periphery of the gland arranged radially around a narrow intercellular space (Fig. 2D).

The epithelial cells of the thyroid mass were closely packed without distinct cell limits. They contained oval to round vesicular nuclei, showing slight irregularity, and some of them contained dense chromatin material. The first appearance of the primitive blood capillaries invading the periphery of the thyroid mass from the surrounding highly vascular mesenchymal tissue began at this stage (Fig. 2D).

In rabbit embryos on 16 days old: The primitive thyroid gland reached its final location just caudal to the laryngeal cartilages on either side of the upper part of the primitive trachea. It was flanked laterally by the primitive carotid arteries and primitive vagosympathetic nerve trunk. The developing thyroid gland exhibited its final shape; formed from two lobes connected by a narrow isthmus that consisted of a thin layer of cell cords invested by mesenchymal tissue. The Ultimobranchial-Parathyroid IV complex had already fused dorsomedially with the primitive thyroid mass. But, they remained as separate entities clearly distinguishable from each other. (Fig. 3A).

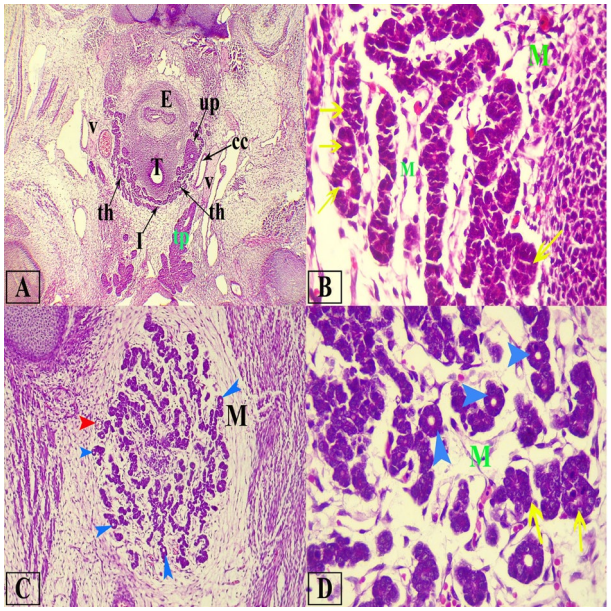

Fig. 3. Photomicrographs of (A) cross section of 16 days old rabbit embryo H&E stain, 4X; (B) high magnification to (A) H&E stain, 40X; (C) longitudinal section of 18th days old rabbit fetus H&E stain, 10X and (D) high magnification to (C) H&E stain, 40X showing, the two primitive thyroid lobes (th) connected by a primitive narrow isthmus (I) on either side of the primitive trachea (T) and esophagus (E); flanked laterally by the primitive carotid arteries (cc), primitive vagosympathetic nerve trunk (V) and ventrolaterally by thymus-parathyroid III complex (tp); ultimobranchial-parathyroid IV complex (up) fused dorsomedially within the thyroid primordium; radially arranged cell cords with intercellular spaces (yellow arrows); highly vascular mesenchymal tissue (M); immature primitive follicles (blue arrowheads); primitive blood vessels (red arrowhead) within the surrounding mesenchymal tissue.

Also, the cellular proliferation was numerously increased within the primitive thyroid gland which was invested in a large amount of the highly vascular mesenchymal tissue. The radially arranged cell cords with intercellular spaces could be observed within the whole primitive thyroid gland. Few groups of cells were still arranged as cell clusters within it. The thyroid precursor cells nuclei reduced in size and contained moderately dense chromatin material with indistinct nucleoli (Fig. 3B).

In rabbit fetuses on 18 days old: The primitive thyroid lobe progressively increased in size and became ellipsoid in shape. The intercellular space transformed into a tiny lumen in each cell group of the radial cell cords, particularly at the periphery of the primitive thyroid gland. The primitive follicular cells became arranged in a single layer around this lumen forming the immature primitive follicles (Fig. 3C and 3D).

The immature mesenchymal connective tissue cells began to circumscribe the primitive thyroid gland, provided with spindle shaped nuclei, and contained primitive blood vessels forming the primitive thyroid capsule. The surrounding mesenchymal tissue with abundant primitive blood capillaries numerously invaded the primitive thyroid parenchyma forming the primitive interlobular connective tissue. The latter gave it the appearance of anastomosing chains of radial cell cords and primitive follicles (Fig. 3C). The immature follicular cells of the primitive follicles exhibited distinct cell boundaries with moderately darkly stained nuclei and scanty eosinophilic cytoplasm (Fig. 3D).

In rabbit fetuses on 20 days old: The primitive highly vascular connective tissue capsule numerously extended into interior of the thyroid parenchyma dividing it into ill-defined lobules, in the form of fine septa. The primitive follicles with narrow empty lumina progressively increased particularly at the periphery of the thyroid parenchyma and were variable in size. Also, a few radially arranged cell cords with intercellular spaces persisted at the center of the gland. The primitive nerve trunk and the primitive cranial and caudal thyroidal vessels could be observed penetrating the primitive capsule of the gland at the cranial and caudal pole, respectively (Fig. 4A). The epithelial cells of immature primitive follicles appeared with darkly stained oval to round nuclei and more eosinophilic cytoplasm in their apical parts (Fig. 4B).

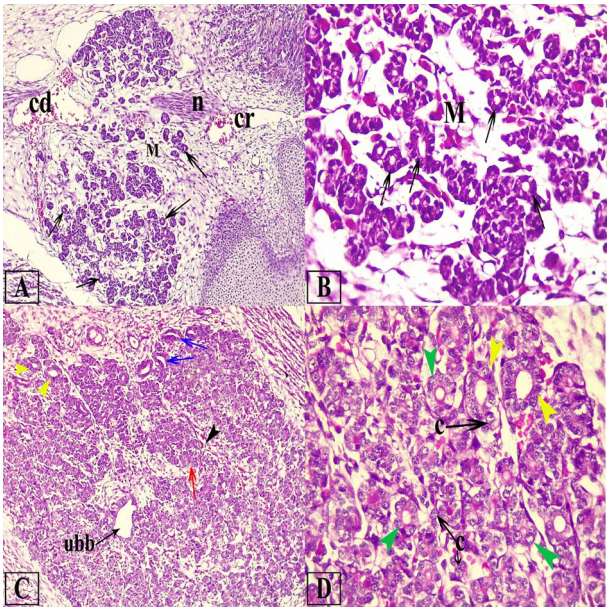

Fig. 4. Photomicrographs of (A) longitudinal section of 20 days old rabbit fetus H&E stain, 10X; (B) high magnification to (A) H&E stain, 40X; (C) longitudinal section of 22nd days old rabbit fetus H&E stain, 10X and (D) high magnification to (C) H&E stain, 40X showing, primitive follicles with narrow empty lumina (black arrows); highly vascular immature connective tissue (M); primitive cranial (cr) and caudal (cd) thyroidal vessels and a primitive nerve trunk (n) penetrating the primitive thyroid capsule; well-defined thyroid lobules (red arrow); primitive interlobular vessels (black arrowhead); Ultimobranchial cyst (ubb); large sized transitional follicles (yellow arrowheads); small and medium sized follicles (green arrowheads); slightly irregular follicles (blue arrows); Parafollicular cells (c).

In rabbit fetuses on 22 days old: The thyroid parenchyma was organized into well-defined lobules and the interlobular connective tissue septa contained primitive interlobular vessels. The developing follicles tremendously increased throughout the whole thyroid parenchyma and could be considered a transitional type between the primitive follicles and the definitive ones (Fig. 4C).

The Transitional follicles arranged into small and medium sized follicles predominated at the center of the gland and large follicles at the periphery. The developing thyroid follicles at this stage were generally round to oval, and slightly irregular shaped ones occurred in small number near the surface of the developing gland. The Ultimobranchial remnants and cysts were incorporated within the center of the developing thyroid parenchyma surrounded by the thyroid transitional follicles (Fig. 4C).

The large sized transitional follicles were lined by high cuboidal follicular cells. While the small and medium sized follicles were lined by low cuboidal cells. The follicular cells showed more eosinophilic material in their apical parts with faintly eosinophilic traces of secretory substances within their lumina. Very few pale staining cells began to be demonstrated among the follicular cells, probably the parafollicular cells. The latter cells contained very little cytoplasm and round nucleus with size almost equal to or slightly larger than the follicular cells (Fig. 4D). There was PAS positive thick rim of pre colloid material in the apical parts of the follicular cells bordering the lumina of a few isolated follicles within the gland (Fig. 5A).

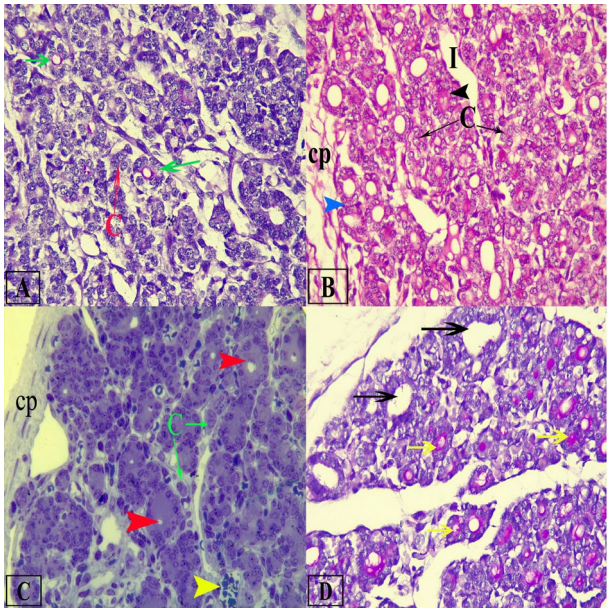

Fig. 5. Photomicrographs of (A) longitudinal section of 22 days old rabbit fetus PAS stain, 40X; (B) cross section of 24th days old rabbit fetus H&E stain, 40X; (C) cross section of 26th days old rabbit fetus toluidine blue stain, 40X and (D) cross section of 26th days old rabbit fetus PAS stain, 40X showing, follicles with definite rim of pre colloid material (green arrows); Parafollicular cells (C); wide interlobular septa (I); primitive capsule (cp); the cells of follicles containing colloid (black arrowhead) more eosinophilic than of the follicles depleting colloid (blue arrowhead); typical colloid (red arrowheads) stored in the most thyroid follicles; extensive capillary network (yellow arrowhead) around the follicles; PAS positive reaction in the stored colloid (yellow arrows) and empty irregular follicles (black arrows) without any PAS positive material.

In rabbit fetuses on 24 days old: The transitional follicles verging on maturity assumed an appearance similar to the definitive follicles with small amount of colloid material in some follicles. The thyrocytes contained round vesicular nuclei with distinct nucleoli, and their cytoplasm were more eosinophilic in the follicles with colloid material than the follicles depleting colloid. The parafollicular cells were seen paler stained cells with central round nuclei and larger than the surrounding follicular cells. They appeared either singly or in small groups of two to three cells in interfollicular location between the developing thyroid follicles (Fig. 5B).

In rabbit fetuses on 26 days old: The thyroid follicles significantly increased in size and number. Also, the gland became well vascularized. Concurrent with the final differentiation of the follicular cells, their secretory substances became typical colloid stored in the most thyroid follicles. The follicular cells appeared with round to oval vesicular nuclei and some cells contained darkly stained nuclei. There was PAS positive reaction in the stored colloid and apical part of follicular cells of empty follicles. While empty irregular follicles did not give any PAS positive reaction neither in their lumina nor in cytoplasm of their follicular cells (Fig. 5C and 5D).

In rabbit fetuses on 28 days old: The thyroid follicles at this age were considered as the definitive follicles, as they stored a typical colloid material which significantly increased throughout the whole gland. The colloid became more vacuolated and stained strongly with Eosin and PAS. The definitive follicles were arranged in form of round to oval follicles of various sizes on a thin layer of basement membrane. The colloid was less vacuolated and densely eosinophilic in small follicles than the larger ones. The thyrocytes ranged from simple cuboidal in large follicles with abundant colloid to high cuboidal in smaller follicles with moderate amount of colloid. The fibrous connective tissue capsule became more pronounced around the thyroid gland and the collagen fibers could be weakly demonstrated within the capsule by Masson's Trichrome stain (Fig. 6).

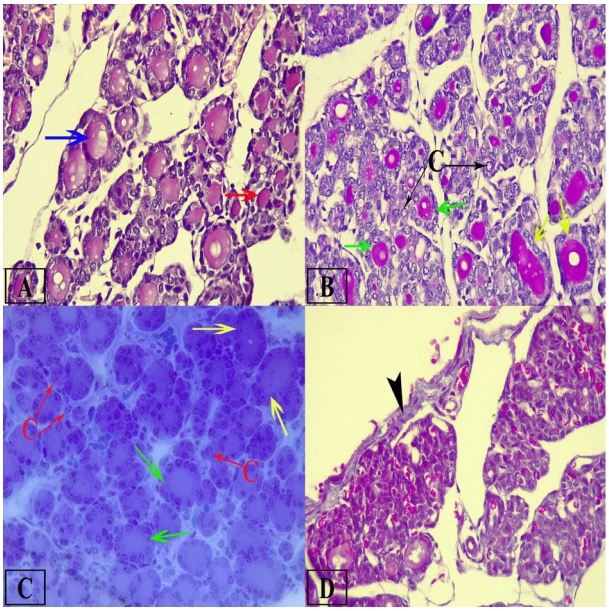

Fig. 6. Photomicrographs of cross section of 28th days old rabbit fetus (A) H&E stain, 40X; (B) PAS stain, 40X; (C) toluidine blue stain, 40X and (D) Masson's Trichrome stain, 40X showing, less vacuolated and densely eosinophilic colloid in small follicles (red arrow) than the larger ones (blue arrow); the thyrocytes ranged from low cuboidal in follicles with abundant colloid (yellow arrows) to high cuboidal in follicles with moderate amount of colloid (green arrows); parafollicular cells (C) and the collagen fibers weakly demonstrated within the capsule (black arrowhead).

In rabbit fetuses on the day of birth: The thyroid gland had a fully differentiated architecture. The follicular cells within the thyroid tissue at this stage showed different levels of activity. The resting follicles with abundant darkly eosinophilic less vacuolated colloid, lined by simple flattened squamous cells. The moderately active follicles with a reasonable amount of eosinophilic colloid, lined by simple cuboidal epithelium. The highly active follicles with scanty, paler, and more vacuolated colloid, lined by high cuboidal epithelium. The paler larger parafollicular cells could be significantly demonstrated in between the follicular cells that contained round to oval vesicular nuclei (Fig. 7A).

The connective tissue stroma became mature represented by a thin fibrous connective tissue capsule composed mainly of collagen fibers which were numerously demonstrated by Masson's Trichrome stain (Fig. 7B). Also, a thin network of interfollicular connective tissue made up mainly of reticular fibers that were detected by Gomori's Silver Impregnation stain (Fig. 7C).

Histometrically, the size of all types of thyroid follicles increased gradually with a corresponding decrease in interstitial connective tissue percentage with advancement of age from 20 days old rabbit fetuses till the day of birth (Fig. 8).

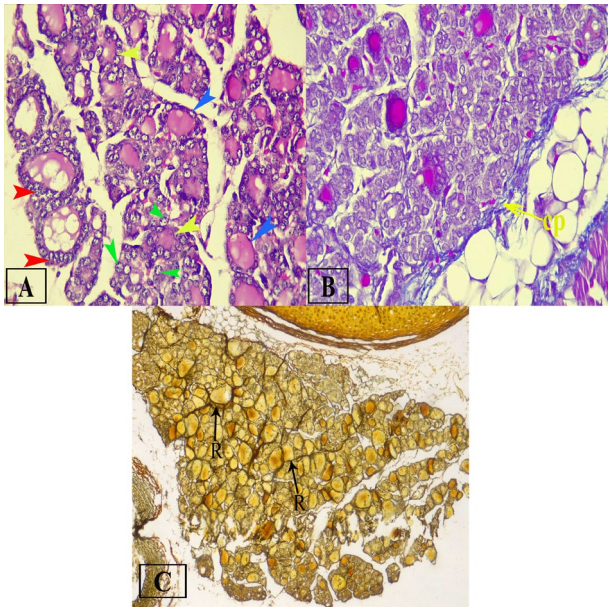

Fig. 7. Photomicrographs of cross section of rabbit fetus at the day of birth (A) H&E stain, 40X; (B) Masson's Trichrome stain, 40X and (C) Gomori's silver impregnation stain, 40X showing, the resting follicles (blue arrowheads) with simple flattened squamous cells; the moderately active follicles (yellow arrowheads) with simple cuboidal epithelium; the highly active follicles (red arrowheads) with high cuboidal epithelium; parafollicular cells (green arrowheads); the collagen fibers strongly demonstrated within the capsule (cp) and reticular fibers (R) between the follicles.

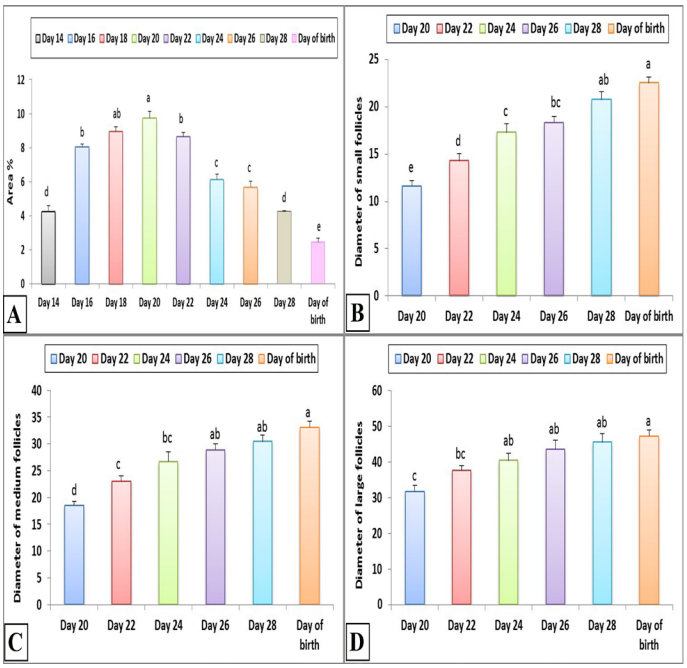

Fig. 8. (A) A bar graph showing gradual increase in the area percentage of rabbit embryos and fetuses thyroid gland interstitial connective tissue from the 14th till 20th days of gestation. Then, gradual decrease from the 22nd day till the day of birth. (B), (C), and (D) Bar graphs showing gradual increase in diameters of small, medium, and large sized follicles of rabbit fetuses thyroid gland, respectively from the 20th day of gestation till the day of birth (Mean ± SEM) (NO. = 10).

Discussion

The present study aimed to clarify the prenatal development of the rabbit thyroid gland at different gestational periods. The thyroid anlage developed as endodermal thickening in the pharyngeal floor in between the first and second branchial arches on 9 days old rabbit embryos. This thickening occurred as a result of assembly of thyroid progenitor cells in a restricted region of the endoderm switching it from a simple to multilayered epithelium. This result was in a line with that found by Waterman and Gorbman (1956) in rabbit. While Kawaoi and Tsuneda (1985) in rat mentioned this result on the 11th day of gestation and Davies *et al.* (2011) in mouse showed this finding at embryonic day (E)8. Meanwhile, Taniguchi *et al.*, (1990) in golden hamster detected this result between day 9-10 of gestation. Also, Arrangoiz *et al.* (2018) and Pradhan *et al.* (2021) in human reported this result during the second and third week of fetal life.

Our result followed that mentioned by De Felice and Di Lauro (2016) in mice, as they found that the thickening of endodermal epithelium in the foregut had been suggested to be an essential event in the generation of signals required for the continuation of thyroid gland organogenesis.

On 10-12 days old rabbit embryos, the formation of thyroid bud from the endodermal cells of thyroid anlage occurred concomitantly with the proliferation of thyroid progenitor cells. This signified by the thyroid anlage becoming more crowded with cells and appeared as a pseudostratified endodermal outpouching penetrated the underlying mesenchymal tissue. The thyroid bud was connected to the pharyngeal endoderm by a thyroglossal duct and located cranial to the dorsal aorta. This result corresponded with the results of Waterman and Gorbman (1956) in the same animal except that they detected this finding on the 10th day of gestation.

Moreover, Fagman *et al.* (2006) and Kameda (2016) in mouse showed the thyroid primordium at Embryonic day (E) 9 as an endodermal bud originating from the ventral floor of the second pharyngeal arch. Then, it moved caudally to be located cranial to the aortic sac at E11.5. While De Felice and Di Lauro (2016) and Nilsson and Fagman (2017) detected the emergence of the thyroid bud at E 9.5 in mouse and the former authors showed this result at E 26 in human.

The present study revealed that by the end of this stage on 12 days old rabbit embryos, the thyroid bud incompletely detached from the pharyngeal endoderm by gradual degeneration of the thyroglossal duct which was a transient embryonic structure. Then one day later, it was completely separated from the pharyngeal floor and had migrated further caudally to reset as a cap like structure on the primitive common carotid artery. This result came in parallel with those observed by Waterman and Gorbman (1956) in rabbit except that, they observed this finding on the 11th day of gestation. Furthermore, Sugiyama (1941) in albino rat demonstrated the thyroid primordium in the 6 mm stage as a long ellipsoid mass with a small cavity located on the truncus arteriosus. Also, De Felice and Di Lauro (2016) and Nilsson and Fagman (2017) in mouse detected this result at E10.5 and the former authors in human showed degeneration of the thyroglossal duct around E 37.

On the other hand, Stewart and Rizzolo (2012); Policeni *et al.* (2012) and Khatawkar and Awati (2015) in human clarified that at the fifth week of gestation, the thyroglossal duct degenerated but its distal part might persist as a pyramidal lobe extended superiorly from the isthmus.

Our result showed the thyroid bud on 13 days old rabbit embryos as a solid endodermal mass. Its epithelial cells had round to oval vesicular nuclei and surrounded by a highly vascular undifferentiated mesenchymal tissue. This finding concurred with results of Togari *et al.* (1952) in rabbit on the 14th day of gestation.

During the caudal migration of the primitive thyroid gland on the 14th day of gestation, the surrounding vascular mesenchymal elements invaded its parenchyma preparation to differentiation of various cell arrangements. The solid cell clusters generally at the center of the gland and anastomosing solid cell cords with the appearance of a few radially arranged cell cords around a narrow intercellular space at the periphery. Two days later after a transient phase of active migration, the primitive thyroid gland reached its final location just caudal to the laryngeal cartilages on either side of the upper part of primitive trachea. Also, the thyroid mass began to differentiate into two lobes with a narrow isthmus. The latter consisted of a thin layer of cell cords invested within a highly vascular mesenchymal tissue.

The latter findings were also achieved by Togari *et al.*, (1952) in rabbit on the 14th-16th days of gestation and by Waterman and Gorbman (1956) in the same animal on the 15th-16th days of gestation. However, Soliman *et al.*, (2005) in white New Zealand rabbit observed the thyroid primordium on the 12th day of gestation as two oval masses of irregular cell cords and clumps close to the tracheal anlage. The gland assumed the bilobed form with a narrow isthmus at the 18th day of gestation. Also, these outcomes showed by Sugiyama (1941) in albino rat at 10-12 mm stage and in mice at 8-11 mm stage. Moreover, Davies *et al.* (2011); De Felice and Di Lauro (2016) and Nilsson and Fagman (2017) in mouse detected these findings at E13.5. However, Taniguchi *et al.* (1990) in golden hamster detected an isthmus consisting of several layers of cell cords between days 10-11 of gestation.

The present findings agreed with those mentioned by Kameda (1984) in rabbit. He observed the primitive thyroid cells of rabbit embryos of about 16 days of intrauterine period were arranged in irregular clusters and radially arranged cell cords, supported with loose undifferentiated mesenchymal tissue.

The immature primitive follicles began to appear at the periphery of the thyroid lobes on the 18 day prenatally. Then, on the 20th day of gestation, the variable sized primitive follicles with a narrow empty lumen progressively increased throughout the thyroid parenchyma. Their epithelial cells appeared with darkly stained oval to round nuclei. Their cytoplasm was more eosinophilic in their apical parts implying the forerunning of

secretory function unless the secretory substances failed to be found. This coincided with consequences observed by Togari *et al*. (1952) in rabbit on the 16th-19th days of gestation and by Kameda (1984) in rabbit on 16 days old rabbit fetuses. Nevertheless, Soliman *et al*. (2005) in white New Zealand rabbit observed the small primitive follicles with narrow empty lumina on 14 days old rabbit fetuses.

Moreover, Kawaoi and Tsuneda (1985) in rats detected few primitive follicular structures between the thyroid epithelial cells on the 17th-18th day of gestation. Kameda (2016); De Felice and Di Lauro (2016) and Nilsson and Fagman (2017) in mice observed small rudimentary follicles within thyroid parenchyma at E15.5. Taniguchi *et al*. (1990) in golden hamster noticed follicular structure with small empty lumen at 15 days of prenatal life. De Felice and Di Lauro (2016) and Gevers *et al*. (2016) detected primary follicles in the human fetal thyroid after the 11th week of pregnancy. While Bande *et al*. (2018) in human reported that the arrangement of thyroid follicles at the periphery of the gland began from the 13th week of gestation.

On the 22nd day of intrauterine life, the transitional follicles developed from the primitive ones. They arranged into round to oval small and medium sized follicles predominated at the center of the gland and large follicles with slightly irregular shaped ones at the gland periphery. The follicular cells of large follicles were high cuboidal and of the small and medium ones were low cuboidal cells. This result agreed with Togari *et al*. (1952) in rabbit but on the 19th-21st days of gestation. While Soliman *et al*. (2005) in white New Zealand rabbit observed well developed but inactive follicles in 20-22 days old fetuses.

The thyroid follicles continued to significantly increase in size and number throughout the thyroid gland till appearance of well-organized definitive follicles on 28 days old fetuses. They were arranged in form of round to oval follicles of various sizes on a thin layer of basement membrane with follicular cells ranged from simple to high cuboidal. This finding came in accordance with consequences observed by Togari *et al*. (1952) in rabbit but on the 21st-22nd days of gestation. While Soliman *et al*. (2005) in white New Zealand rabbit observed these findings in 24-26 days old fetuses. Similar result was mentioned by Das *et al*. (2017) in human on the 15th week prenatally.

Meanwhile, the typical follicles demonstrated in rat by Sugiyama (1941) at the 25 mm stage and by Kawaoi and Tsuneda (1985) on the 21st day of gestation. Also, this finding was observed in mice by Sugiyama (1941) at the 18 mm stage and by De Felice and Di Lauro (2016) at E17-18.

Our result was agreed upon by Fagman *et al*. (2006) and Gevers *et al*. (2016) who explained that the onset of functional differentiation of thyroid follicular cells with the formation of different sized follicles occurred only once thyroid migration was complete.

The present work revealed that the first appearance of PAS positive thick rim of pre colloid material in apical parts of the follicular cells in few follicles within the gland occurred on the 22nd day of prenatal life. This considered the first morphological evidence of functional differentiation of the thyrocytes. This result was contradicted by Togari *et al*. (1952) in rabbit, who detected traces of faintly eosinophilic secretory material within the cavities of few transitional follicles from 19 days of gestation onward. Moreover, Kameda (1984) in the same animal recorded the first appearance of PAS-positive colloid droplets in small cavities among cell clusters of primordial follicles in 18 days old fetuses. While Soliman *et al*. (2005) in white New Zealand rabbit observed the colloid within the lumina of some central follicles between the 24th and 26th days of gestation. On the other hand, Khatawkar and Awati (2015); De Felice and Di Lauro (2016) and Arrangoiz *et al*. (2018) in human demonstrated that, the colloid began to appear in the thyroid follicular structures during the 11th week of pregnancy. While Bande *et al*., (2018) in human found a thin rim of colloid on the 14th week prenatally.

On the 24th day prenatally, small amount of colloid material stored within some follicles verging on maturity as their cells attained a secretory activity. Concurrent with the final differentiation of the follicular cells, their secretory substances became typical colloid stored in the most thyroid follicles on 26 days old. The typical colloid material became more vacuolated and stained somewhat strongly with Eosin and PAS and significantly increased throughout the whole gland from 28 days old fetuses till the day of birth. Though, Togari *et al*. (1952) in rabbit detected the typical colloid on the 21st-22nd days of gestation.

Also, Waterman and Gorbman (1956) in rabbit observed the first few small follicles with colloid in 16-17 days of pregnancy. Around 20-22 days of prenatal life, well organized follicles with colloid were seen often prominently in the periphery of primordium. Moreover, Kameda (1984) in rabbit considered the true onset of thyroid function began on the 22nd day of gestation as the colloid stored in considerable amount within the follicles. On the other hand, Kawaoi and Tsuneda (1985) in rats detected PAS reactive colloid material within follicles lumina on the 19th day of intrauterine life. While Bande *et al*. (2018) in human detected the vacuolated colloid in all follicles particularly in peripheral follicles between 17-20 weeks of pregnancy.

The present findings were similar to the statements of Togari and Sugiyama (1951) in rabbit as they revealed the Ultimobranchial body as Ultimobranchial-Parathyroid IV complex lay dorsolaterally close to the developing thyroid gland on the 14th day of gestation. While Soliman *et al*. (2005) in white New Zealand rabbit observed this finding on the 20th days old fetuses, Kimura (2014) and De Felice and Di Lauro (2016) in mice at E13 and in human at E40. Then, the Ultimobranchial body dispersed dorsomedially within the thyroid tissue on the 16th day of gestation. Nevertheless, Soliman *et al*. (2005) in white New Zealand rabbit observed this on the 22nd day prenatally, Kimura (2014) and De Felice and Di Lauro (2016) in mice at E13.5 and in human at E55. Later, it gave thyroid like follicles and cysts incorporated within the developing thyroid parenchyma surrounded by the thyroid transitional follicles on the 22nd day of gestation. Though, Soliman *et al*. (2005) in white New Zealand rabbit observed this on 26 days old fetuses, Kimura (2014) and De Felice and Di Lauro (2016) in mice at E15.5.

Very few parafollicular cells began to disseminate among follicular cells on 22 days old fetuses. Later, these cells were seen paler stained with central round nuclei and larger than the surrounding follicular cells. They appeared either singly or in small groups of two to three cells in interfollicular location. This finding disagreed with Soliman *et al*. (2005) in white New Zealand rabbit, who recorded the Parafollicular cells for the first time between the thyroid cord cells on 12 days old fetuses and between the epithelial cells of the ultimobranchial body on 20 days old fetuses. Meanwhile, Togari *et al*. (1952) in rabbit did not detect Parafollicular cells prenatally. Moreover, Kimura (2014) and De Felice and Di Lauro (2016) detected the parafollicular cells in mice at E14.5. Also, Das *et al*., (2017) in human reported that, the parafollicular cells were seen as earlier as by the 14th week of pregnancy either interfollicular or intrafollicular location.

The present study on the 14th day of prenatal life revealed that, the surrounding vascular mesenchymal elements began to invest the thyroid parenchyma. Then on the 18th day of gestation, mesenchymal connective tissue cells with elongated nuclei began to surround the thyroid primordium suggesting the primitive capsule. On 20 days old fetuses, the highly vascular surrounding mesenchyme numerously extended into the thyroid parenchyma dividing it into ill-defined lobules with intervening undifferentiated connective tissue septa. In rabbit fetuses of 22 days old, the thyroid parenchyma was organized into well-defined lobules and interstitial connective tissue converted into young connective tissue. Finally, at the 28th day of gestation, the fibrous capsule became more pronounced around the thyroid gland and composed mainly of collagen fibers which were numerously demonstrated at the day of birth.

The latter findings contradicted by the consequences mentioned by Togari *et al*. (1952) in rabbit. They showed that, the mesenchymal cells surrounded the thyroid primordium in layers and invaded its parenchyma at 15 days old fetuses. Then at 18 days prenatally, the mesenchymal cells contained elongated nuclei and at 20 to 21 days old fetuses, they converted into young connective tissue cells. They became more mature and densely arranged with the appearance of follicles at 21 to 22 days of gestation.

Moreover, Sugiyama (1941) cleared that in the 15 mm stage of the albino rat and in the 11 mm stage of the mouse embryo, the surrounding mesenchyme began to be arranged in lamellae. The surrounding mesenchyme entered the thyroid parenchyma dividing it into several blocks in the 12 mm stage and 11 mm stage in the albino rat embryo and the mouse embryo, respectively. Kameda (2016) in mice mentioned that mesenchymal cells began to colonize the developing thyroid at E14.5.

Nevertheless, Das *et al*. (2017) in human observed a thin connective tissue capsule with blood vessels around the developing thyroid gland at 14 weeks of gestation. Then, at 15 weeks the capsule became thicker and was sending connective tissue septa dividing the parenchyma into ill-defined lobules. While Bande *et al*. (2018) in human reported that on the 12th week prenatally.

Our result revealed that the thyroid stroma consisted of a thin network of interfollicular connective tissue made up mainly of reticular fibers that became more prominent at the day of birth. This was confirmed by Togari *et al*. (1952) and Soliman *et al*. (2005) in white New Zealand rabbit. While Sugiyama (1941) detected that in albino rat at the 25 mm stage, and in mouse at 18 mm stage.

The present investigations agreed with Nilsson and Fagman (2017) that the gradual enlargement of the thyroid gland till the day of birth attributed to the generation of new follicles and a gradual increase in the size of individual follicles.

The present results revealed that the size of all types of follicles increased gradually with a corresponding decrease in interstitial connective tissue percentage with advancement of age from the 20 days old rabbit fetuses till the day of birth. The long diameters of follicles reached its maximum size at the day of birth. This disagreed with that observed by Togari *et al*. (1952) in rabbit, who mentioned that the thyroid follicles

diameter increased throughout prenatal life and reached its maximum size shortly before the birth on the 28th day of gestation but reduced at the day of birth.

We observed that the primitive interstitial connective tissue percentage was little on 14 days old embryos and increased gradually till reaching its maximum level on the 20th day prenatally then, began to decrease gradually again till reaching its minimal level at the day of birth. Our result came in accordance with Das *et al.* (2017) in human who observed very little intervening connective tissue at 14 weeks prenatally and increased gradually then, began to decrease at 18-20 weeks of gestation and reached its minimal level at 28 weeks of pregnancy.

Conclusion

Our study clarified that; the thyroid anlage began to develop as endodermal thickening in the pharyngeal floor at 9 days old rabbit embryos. The beginning of differentiation of thyroid mass into two lobes with a thin isthmus began on the 16th day of prenatal life, accompanied by folliculogenesis and differentiation of progenitor cells to hormone producing thyrocytes. The first evidence of follicular organization appeared on the 20th day of gestation with many small non secretory follicles disseminated within the gland. The beginning of progenitor cells differentiation to secrete precolloid substances occurred at 22nd days old fetuses. The demarcation of well-organized thyroid stroma occurred on the 28th day prenatally. The area% of the primitive interstitial connective tissue began little at 14 days old embryos and increased gradually till its maximum level on the 20th days prenatally then, began to decrease gradually again till reaching its minimal level at the day of birth. The long diameters of all types of follicles increased gradually with a corresponding decrease in interstitial connective tissue percentage with advancement of age from 20 days old rabbit fetuses till became maximum at the day of birth.

Acknowledgments

The authors thank Anatomy and Embryology Department members, Zagazig University, Egypt, for their cooperation.

Conflict of interest

The authors declare that they have no conflict of interest.

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