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EC CLINICAL AND EXPERIMENTAL ANATOMY
Research Article

Gene and Marker Stem Cells Expression in Mouse Thyroid Gland against Age Changes and the Prophylactic Role of Selenium

Laila M Aboul-Mahasen Morsy[1,4]*, Mai M Ashgan[2] and Saleh A AlKarim[2,3]

[1]*Anatomy Department, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia*

[2]*Biology Departments, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia*

[3]*Embryonic and Cancer Stem Cells Research Group, king Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia*

[4]*Anatomy Department, Faculty of Medicine for Girls, Al-Azhar University, Cairo, Egypt*

***Corresponding Author**: Laila M Aboul-Mahasen Morsy, Professor, Anatomy Department, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia and Anatomy Department, Faculty of Medicine for Girls, Al-Azhar University, Cairo, Egypt.

Received: March 12, 2020; **Published:** April 10, 2020

Abstract

Background/Aim: The expression of stem cells in developing and aged thyroid tissue is still not clearly detected. This study is aimed to investigate the stem cell expression in mouse thyroid gland at different ages from newborn to old age and the possible prophylactic effect of selenium from development of aging-associated stem cell defects.

Materials and Methods: 60 laboratory mice were used and divided into six groups at ages of (one day, 15 days, 30 days, 2 - 3 months, 12 - 24 months and selenium treated 12 - 24 months). The treated group were given selenium (5 mcg) orally for 20 successive days. Blood were harvested from all groups for assessment of thyroid function tests (TSH, T3 and T4). Slices of thyroid glands were prepared for P63 immunohistochemistry and for qRT-PCR gene expression pattern for genes *ABCG2, GNL3, OCT4* and paired box *PAX8* genes against reference actin beta (ACTB) in mice. Real time quantitative PCR data were analyzed by Livak method.

Results: The immunohistochemical and RT-qPCR results revealed gene expression of stem cells in all ages but with lower expression in aged group than those in adult group. In the aged group treated with selenium, the stem cells expression is improved. Relative quantification for *OCT4* and the fold changes in comparative differential expression revealed a significant upregulation only in the aged group. However, gene expression in the selenium treated aged group gave nearly the same expression as those of the adult and 30 days old groups.

Conclusion: As the biochemical results in selenium treated aged group confirmed the immunohistochemical and RT-qPCR gene expression results, So, it could be concluded that the stem cells have been expressed in the mouse thyroid tissues at all ages and the possible prophylactic effect of selenium on the aged stem cells had been detected.

Keywords: *Stem Cells; Postnatal Developing Thyroid Tissue; Aging Process; Selenium; Reference Gene (ACTB); Stem Cell Marker P63; ABCG2; GNL3; OCT4; PAX8 Genes*

Abbreviations

C Cells: Parafollicular Cells (PC); SCNs: Solid Cell Nests; SPs: Side Populations; Tg Marker: Thyroglobulin Marker; *ABCG2* Gene: ATP-Binding Cassette Family G 2 Gene; *GNL3* Gene: Guanine Nucleotide-Binding Protein-Like 3 Gene; *OCT4* Gene: Octamer Transcription Factor Family 4 Gene; PAX 8 Gene: Paired Box (PAX) Family 8

Introduction

Thyroid stem cells were detected as cell populations called solid cell nests (SCNs) and side population cells (SP). SCNs were stem cells of endodermal origin derived from ultimobranchial body remnant [1]. SCNs were consisted of main and parafollicular cells. Another structures called cystic SCNs were detected [2,3]. It had been supposed that the main cells suggested to be the stem cells. The immunohistochemical pattern of SCNs studied the stem cell role of SCNs main cells and expressed by the common stem cells markers [4]. The main cells of SCNs expressed P63 which highly detected in progenitor/stem cells [5]. P63 was highly detected in thyroid SCNs and that cells showed a stem cell like character [6,7]. Side population cells were full with cells have stem cell like character as gave highly expression for *ABCG2* [8].

Quantitative RT-PCR gene analysis is one of the most accurate gene analysis methods. Data analysis of PCR is a commonly used technique for the assay of expression of mRNA throughout stem cell studies. DNA (cDNA) is originated from the RNA through the process of reverse transcription and then the data is analyzed by qRT-PCR using reference genes as control [9].

The thyroid Stem cells were genetically expressed by using PCR for expression of stem cell markers (*ABCG2*, *OCT4*, GNL4). *ABCG2* gene was commonly used as stem cell marker for detection of normal stem cells [4,8,10,11], while *OCT4* gene was f used as a marker for undeveloped cells and it was used also for expression of adult stem cells [7,12,13]. *GNL3* protein, is encoded by the *GNL3* gene. It is present inside the nucleolus that connected to P53. It is a marker for many stem cells [8,14-17]. Paired transcription factor paired PAX 8 is a gene associated with thyroid differentiation. It had a crucial role in the tissue and organ development from prenatal to postal period [18,19].

Senile tissues showed a marked gradual decrease in the functional and renewal abilities, which led to alteration in stem cells inside the tissues. Regenerative therapies focused on stem cells and other factors which could improve aging. Great researches concentrated on the advanced methods to direct the aged genes and stem cells [20]. The progression of regenerative therapy that could improve the degenerative changes related to age became important [21].

Selenium is an important micronutrient. Scientists had shown that selenium was essential for maintaining a healthy immune system, hormonal balance and metabolism. The highest level of selenium in the body is present in the thyroid gland. Selenium was used there as a building block of specific proteins called selenoproteins. Some selenoproteins were antioxidants as removing the oxidative radicals that were generated in the process of synthesis of thyroid hormones. Another subgroup of selenoproteins called deiodinases, were crucial for transforming the inactive T4 into the active T3. If selenium level was missed in the body, production of T3 would be lowered and a part of the thyroid gland became non-functional [22]. So, selenium had an important function in preventing the premature ageing and thyroid gland damage [23].

Aim of the Work

While many studies had been expressed the stem cells in adult thyroid glands, nearly no studies were explained the gene expression profile of stem cells in thyroid glands from postnatal age and old age. So, this study investigated the immuno- expression of stem cell marker P63 and assessed the gene expression pattern of stem cells and thyroid differentiation cells on thyroid mouse related to the advancement of age and selenium treatment on gene expression of *ABCG2, GNL3, OCT4* and paired box *PAX8* genes by using RT-qPCR gene expression analysis.

Ethical approval

This study was followed the guidelines and protocols approved by the ethical committee for animal care unit in King Fahd Medical Research Centre, King Abdulaziz University, Saudi Arabia, Jeddah which are in accordance with the Canadian Council on Animal Care guidelines.

Materials and Methods

Mice were taken from the animal experimental unit of King Fahd Medical Research Center (KFMRC), KAU, KSA, Jeddah. Mice were put in good - aerated cages and maintained in a 24°C ± 1°C temperature- controlled room with a 12 hours light/12 hours dark cycle, 55% ± 10% humidity.

Experimental design

Sixty laboratory mice at ages of 1 day, 15 days, 30 days, 2 - 3 months and 12 - 24 months were used. The mice kept at room temperature and received food and water *ad-libitum*. They were divided into 6 groups (10 mice each): Group 1: 1 day old, Group 2: 15 days old, Group 3: 30 days old (prepubertal age), Group 4: 2 - 3 months old (adult age), Group 5: 12 - 24 months old (aged/old age) and Group 6: 12 - 24 months old (selenium treated old age) which were given selenium (5 mcg) by a gastric tube for 20 successive days. Then, blood were taken and collected from all groups and assessed for thyroid function tests included TSH, T3 and T4. Then, the mice anaesthetized and slices of the thyroid glands were taken and processed for histological, immunohistochemical study and for PCR gene expression.

Blood analysis

Blood samples were taken and collected from all groups for the assessment of TSH, T3 and T4 and selenium levels. The enzymatic immuno-assay methods using the following kits for measurements: Rat Thyroid Stimulating Hormone (TSH) Elisa kit (Competitive ELISA) (My BioSource. com. San Diego. USA. Catalog No. MBS726442), Rat Triiodothyronine (T3) ELISA Kit (My BioSource. com. San Diego. USA. Catalog No: MBS016153), Rat thyroxine (T4) ELISA Kit ((My BioSource. com. San Diego. USA. Catalog No. MBS704309) and Rat Selenium-binding protein 1 (SELENBP1) ELISA Kit (WWW.abbexa.com. Cambridge, UK. Catalogue No.:abx259346).

Technique for histological study

Thyroid glands were extracted from mice of different ages, the thyroid specimen were processed for histological examination. They were dehydrated in ascending grades of ethyl alcohol, cleared in benzene, impregnated in paraffin and finally embedded in paraffin wax. The paraffin blocks were cut at 4- 6 um thickness. Thyroid paraffin sections were stained with H&E staining [24].

Technique for immunohistochemical study

Immunohistochemical examination for expression of thyroid stem cells P63 in the different groups [4,7] was done. Recommended Staining Protocol for VENTANA anti-p63 (4A4) with ultra View Universal DAB Detection Kit on a Bench Mark ULTRA instrument. This antibody showed a nuclear staining pattern. It used for identification of the stem cell -like in the thyroid gland [4,24]. Finally, all slides were scanned by Digital pathology slide scanner (Philips Intelli Site Pathology Solution). Photographs were capturing from the scanned slides.

Selenium

As an adequate selenium intake in the diet might not be enough, so 200 mcg (micrograms) per day was the most frequently used dose in studies that obtained beneficial results [25,26]. In this study, Selenium enriched yeast (Brewer's Yeast) tablets was be purchased from (Jamieson pharmaceutical company, Canada). The tablets were powdered and dissolved by distilled water. The dose of Selenium/mouse was calculated according to Nair and Jacob [27]. The supposed body weight of an old mouse is 25 gm, the experimental dose was 200 X 25/1000 = 5 mcg. The aged mice were given selenium (5 mcg) orally by gastric tubes for 20 successive days.

Real-Time-quantitative PCR

RT-qPCR was done at GenaTi Unit at King Fahd Medical Research Centre, King Abdulaziz University.

It was conducted to analyze the gene expression pattern of stem cells and thyroid differentiation cells on thyroid mouse and to analyze the effect of age and treatment on gene expression of *ABCG*, *GNL3*, *OCT4* and paired box (*PAX8*) genes.

Method of RNA extraction

Total RNA was isolated from the cells using column purification technology using RNeasy Mini Kit (Qiagen). Separation of RNA was carried out according to the manufacturer 's instructions (Qiagen).

RNA quantification and assessment of the purity

An aliquot (1 µl) of each sample was used to test the quantity and purity of the RNA on a nanodrop (NANODROP 2000c). The purity was determined at a wavelength A260/A280 and the range between 1.8 to 2 is defined as pure. The samples were frozen at (–80°C) until use or used directly for reverse transcription reaction and gene expression studies.

Reverse transcription reaction

cDNA reverse transcription kit (cDNA Promega) was used for reverse transcription of RNA to complementary deoxyribonucleic acid (cDNA). RNA was taken and mixed with stock solution (1) in table 1 and transmitted to cycler sequencing machine used for denaturation of RNA at 70°C for 5 minutes and preserved on ice, then mixed with 7.7 µl of stock (2) (Table 1) and the reaction run on thermal cycler with the following cycling conditions 25°C for 5 minutes, 42°C for 120 minutes and finally 70°C for 15 minutes. Measurement of cDNA concentration by using nanodrop at a wave length A260/A280.

Components	Volume/reaction
Stock (1)	
Experimental RNA	2 µl
Primer [Oligo (dT) 15	0.5 µl
Random primer (50 µg)	0.5 µl
Nuclease-Free Water	2 µl
Total Volume	5 µl
Stock (2)	
Nuclease-Free Water (to a final volume of 15 µl)	7.3
Buffer (10x)	4 µl
$Mgcl_2$ (25 mM)	1.2 µl
dNTP mix (10 mM)	1 µl
RNase inhibitor (40 u/µl)	0.5 µl
Reverse transcriptase (160 u/µl)	1 µl
Total	15.0 µl

Table 1: *Preparation of master mix of reverse transcription reaction.*

Real Time-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was done using the Fast SYBR Green Master Mix. The data were normalized for gene ACTB. The sequences of primers used in the reaction were listed in table 2 [28-30]. The PCR amplification conditions used were 95°C for 5 minutes followed by 40 cycles of for 95°C 10 seconds and 58°C for 30 seconds.

	Sequences
GNL3	F: 5‘-TTGGGATTTCCATGGGAC- 3‘ R: 5‘- AGTGGGGGAGTTACAAGG- 3‘
OCT4	F: 5’- CAATGAGAACCTTCAGGAG- 3’ R: 5‘-CACGGTTCTCAATGCTAG -3‘
PAX8	F: 5‘- AACCTCTCGACTCACCAG- 3‘ R: 5‘- GGAGCTAGAACTGGAGAG- 3‘
ABCG	F: 5‘-GGAGATTCTTCTGCTGTG -3‘ R:5‘- CTATTACTGGCTTCTCTCC -3‘
ACTB	F: 5‘-CGTGAAAAGATGACCCAG- 3‘ R: 5‘- ACGACCAGAGGCATACAG- 3‘

Table 2*: The sequences of used primers for qRT-PCR reaction*

RT-qPCR Data Analysis

RT-qPCR data was analyzed by ΔΔCT or Livak method [31]. The average cycle threshold (CT) for the endogenous control and each experimental genes was calculated from the raw data generated by RT-PCR Step One System and Data Assist software. The outliers were either omitted from analysis or substituted with means. The graph Pad PRISM software and MS Excel functions were used to perform the analysis and generate the statistical plots of the data.

ΔΔCT or Livak method

At first, we normalized CT of the target gene to the CT of the reference gene, then we normalized ΔCT of the test sample to the ΔCT of the control sample and finally we calculated the relative quantification (Rq) and differential expression (fold change, FC) using following steps:

(i) Calculate ΔCT values of each sample:

ΔCT for Ctrl = CT target gene - CT reference gene

ΔCT for Test = CT target gene - CT reference gene

(ii) Calculate ΔΔCT values for each comparison between two groups

ΔΔCT = ΔCT test sample – ΔCT control sample

(iii) Calculate relative quantification (Rq) values for each comparison between groups:

Relative quantification (Rq) = 2 -ΔΔCT = value*

*Rq >1 represents up-regulation whereas Rq < 1 represents down regulation of gene

(iv) Calculate fold change (differential expression i.e. how many times genes were up or down regulated? for each comparison between

groups. Fold change (FC) was derived from Rq:

For Rq > 1 (FC =Rq), for Rq = 1 (FC = 0) and for Rq < 1 [FC = - (1/Rq)].

Statistical analysis

The data were statistically analyzed. Statistical Package for Social Science (SPSS) version 20 for windows program was applied to analyze the present data. The data were expressed as means +/- standard deviation (SD). Significance versus adult group (G4). Comparison of between groups was performed using One-Way Analysis of Variance (ANOVA) method. Statistical significance was considered at P-value < 0.05.

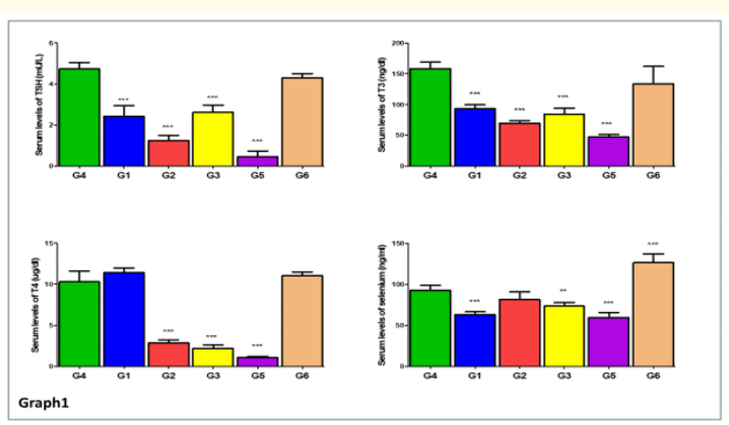
Results

Statistical results of blood analysis

Thyroid function tests revealed that the TSH, T3 and T4 levels were gradually increased till adult age (G4). Whereas their levels revealed a marked statistically decrease at the aged group (G5), they showed a significant increase in the group treated with selenium (G6) as they became nearly as those of the adult group (G4), this indicating the restoration of thyroid activity and the importance of selenium supplementation in old age (Table 3 and Graph 1).

Groups	TSH (mU/L)	T3 (ng/dl)	T4 (ug/dl)	Selenium (ng/dl)
G4 (2 - 3 months)	4.73 ± 0.48	158.33 ± 10.69	10.30 ± 1.31	92.67 ± 6.11
G1 (one day)	2.43 ± 0.52	93.00 ± 7.00	11.43 ± 0.55	63.00 ± 3.61
Significance	0.0001***	0.0001***	0.115	0.0001***
G2 (15 days)	1.25 ± 0.25	70.00 ± 3.61	2.83 ± 0.42	81.33 ± 9.29
Significance	0.0001***	0.0001***	0.0001***	0.102
G3 (30 days)	2.62 ± 0.35	84.67 ± 9.29	2.20 ± 0.39	73.33 ± 4.51
Significance	0.0001***	0.0001***	0.0001***	0.010**
G5 (old age control)	0.46 ± 0.28	47.33 ± 4.047.00	1.09 ± 0.11	59.67 ± 6.11
Significance	0.0001***	0.0001***	0.0001***	0.0001***
G6 (old age treated)	4.31 ± 0.20	133.83 ± 28.22	11.06 ± 1.06	126.33 ± 10.63
Significance	0.157	0.065	0.217	0.0001***

Table 3: *Comparison of serum levels of TSH (mU/L), T3 (ng/dl), T4 (ug/dl) and selenium (ng/dl) in different studied groups versus G4 (2 - 3 months) group. Data were expressed as mean +/- SD. significance versus G4 (2 - 3 months). Significance was made using One Way ANOVA (LSD) test. *: P < 0.05, ***: P < 0.001.*



Graph (1) : *Comparison of serum levels of TSH (mU/L), T3 (ng/dl), T4 (ug/dl) and selenium (ng/dl) in different studied groups versus G4 (2-3 months) group.*

Histological and Immunohistochemical results

Histological examination at one day old mouse (Figure 1a) showing that the thyroid follicles were moderately developed as the wall of each thyroid follicle was lined by a single layer of flat to low cuboid cells, with round nuclei. Few number of parafollicular (C- cells) were seen in the interfollicular space. The C- cells were identified by clear cytoplasm and large nuclei. Most of the cells in the central zone of gland were appeared as irregular solid masses and were surrounded by connective tissue elements, these masses were suggested being the solid cell nests (SCNs). Another cellular solid masses were seen at the periphery of the gland and could be known as side population (SP) cells. SCNs were consisted of two types of cells, the primary or main cells and the C- cells. The main cells, forming the major proportion of SCNs, they were elongated cells with central oval nuclei with strongly eosinophilic cytoplasm. By 15 days old (Figure 1b), some irregular large follicles were showing activity in the peripheral region of the gland. By 30 days old to adult (Figure 1c and 1d), there were a greater incidence of active follicles in the central zone of the gland. The active follicles contained faint stained vacuolated colloid and were lined by one layer of cubical or simple columnar cells indicated the onset of thyroid activity in the synthesis and resorption of thyroglobulin. In the old age (Figure 1e), most of thyroid follicles were inactive, appeared empty or contained accumulated colloid with cracks and were lined by a single layer of simple cuboidal or flat cells. In the old age treated with selenium (Figure 1f) an obvious improvement of the thyroid structure was appeared as many follicles restored their rounded to oval shapes especially in the central region of the lobe. Most of thyroid follicles appeared restoring their activity as the follicular cells appeared cuboidal to low columnar in shape with spherical densely stained nuclei and their lumina showed vacuolated colloid indicating colloid absorption. But there were some follicles still have thick colloid with cracks. Many masses of SCNs, cystic SCNs and SP cells were detected in the parenchyma with apparent mitotic figures in their main cells. In sections stained with P63 (Figure 2), the nuclei of masses of SP cells, main cells of SCNs and cystic SCNs (mixed type) gave strong P63 expression indicating the stem cells like - characters. Some solitary scattered cells gave strong positive P63 in between the follicles were detected. The C-Cells gave negative P63 expression. Finally, the stem cells were strongly P63 expressed in all ages but with lower expression in aged group than those in the adult group. In aged group treated with selenium, the stem cells gave expression nearly like the expression of adult group, indicating the activation of the silent aged stem cells.

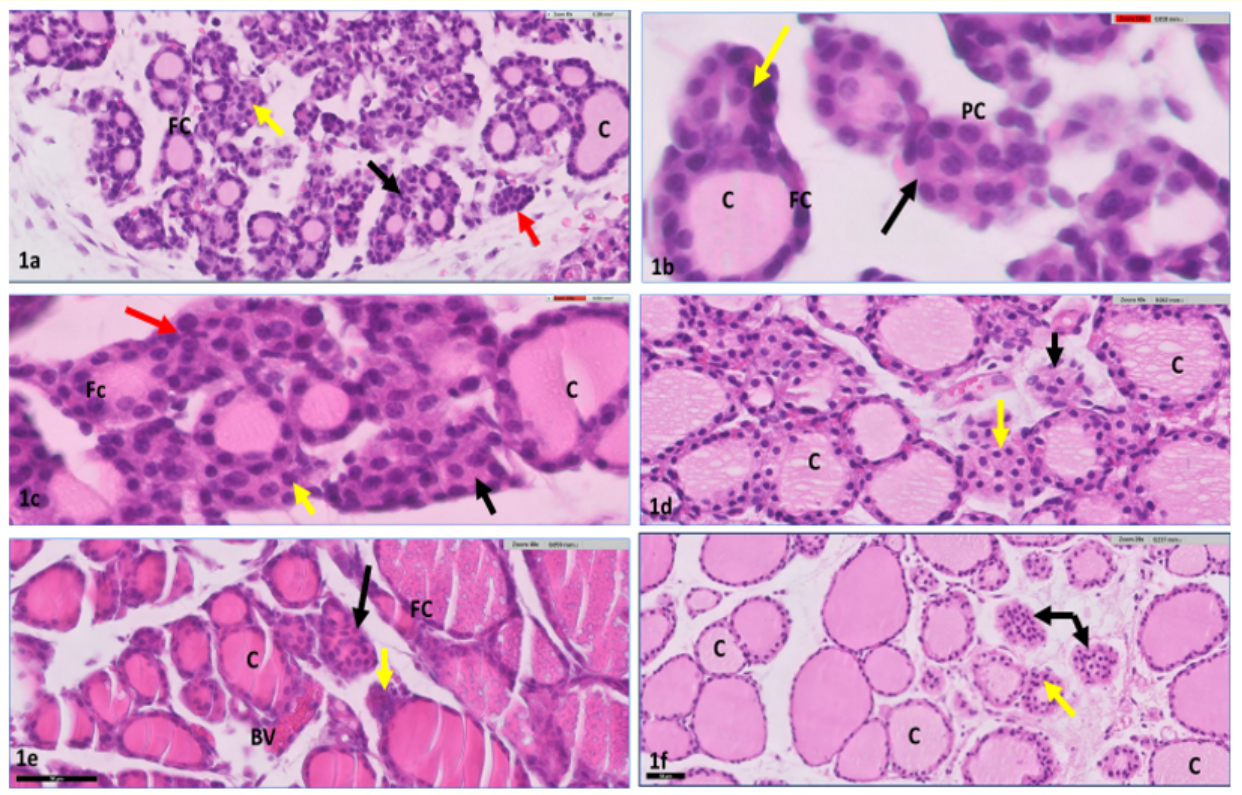


Figure 1: *Photomicrographs of mouse thyroid sections stained with H&E showing*

1a: At 1st day old, moderately developed irregular thyroid follicles in the peripheral region with low cubical follicular cells (FC) and contain colloid (C). Show masses of solid cell nest (black arrow), cystic SCNs (yellow arrow) and side population cells (red arrow). H&E X 40.

1b: At 15 days old, a mass of cystic solid cell nest proliferating a thyroid follicle with high cubical cells (FC) and faintly stained colloid (C). Show also a mass of SCNs (black arrow) contain clear parafollicular C- cells (C). H&E X1000.

1c: At 30 days old, many masses of solid cell nests (black arrow), side population cells (red arrow) and cystic solid cell nest (yellow arrow). Well developed thyroid follicles with regular outlines with low columnar follicular cells (FC) indicating activity and faintly stained colloid (C). H&E X1000.

1d: At 2 - 3 months old, highly active thyroid follicles with vacuolated colloid (C) indicating resorption activity. Notice masses of solid cell nest (black arrow) and cystic solid cell nest (yellow arrow). H&E X 40.

1e: At 12 - 24 months old, irregular thyroid follicles with different shapes, lined with flat follicular cells (FC) and contain thick colloid with cracks (C) indicating the inactivity. Show small masses of SCNs (black) and cystic cell nests (yellow arrow). Congested blood vessel (BV). H&E X40.

1f: At 12 - 24 months old treated with selenium, most of thyroid follicles restore their shape and activity as have faintly stained vacuolated colloid (C). Show any masses of solid cell nest (black arrow) and cystic solid cell nest (yellow arrow). H&E X40.

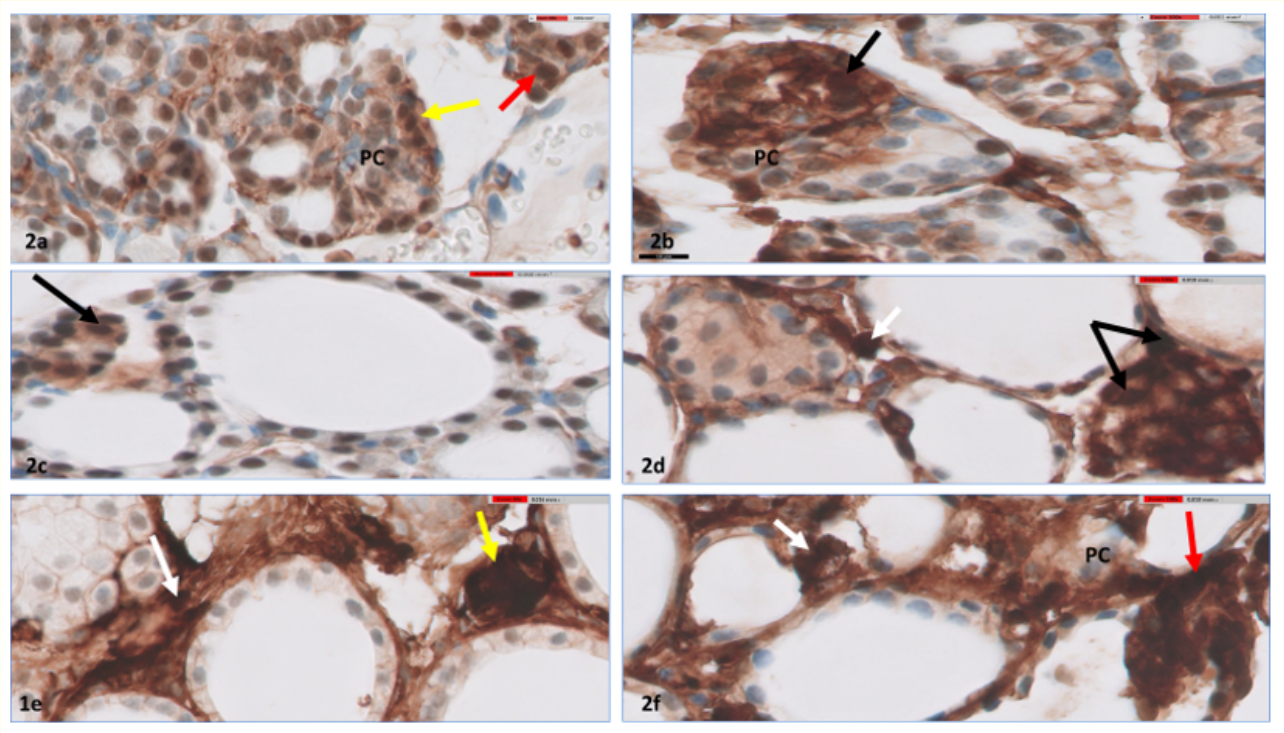


Figure 2: *Photomicrographs of thyroid sections immunostained by P63 showing.*

2a: At 1st day old, strong positive nuclear expression cells of cystic SCNs (yellow arrow) and SPs cells (red arrow) indicating the stem cells like – characters. The C -cells (C) gave negative expression for p63. X1000.

2b: At 15 days old, strong positive nuclear expression for P63 in the main cells of SCNs exhibiting stem cells-like characters. C- cells (C) stained negative for P63.

2c: At 30 days old, strong positive nuclear expression for P63 in the main cells of SCNs (black arrow). X 1000.

2d: At 2 - 3 months old, strong positive nuclear expression for P63 on the main cells of SCN, solitary scattered cells (white arrow). The nuclei showed apparent mitotic activity. X1000.

2e: At 12 - 24 months old, strong positive nuclear expression for P63 to the main cells of SCNs (black arrow). Few solitary scattered cells showing strong nuclear expression for P63. X1000.

2f: At 12 - 24 months old treated with selenium, strong positive nuclear expression for P63 on the main cells of SCN (black arrow) and solitary scattered cells (white arrow). The nuclei showing strongly staining P63 with apparent mitotic activity. The C- cells (PC) stained negative for P63. X1000.

RT-qPCR gene expression pattern

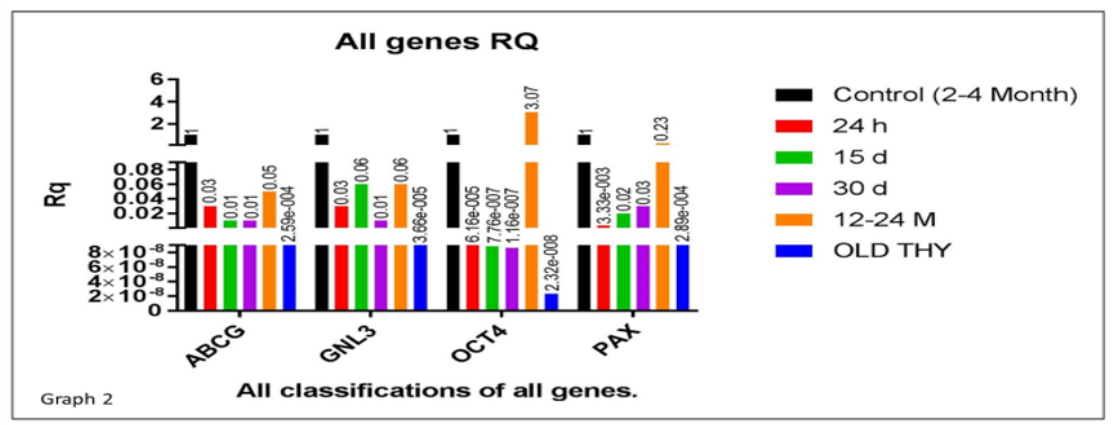
RT-qPCR revealed that SCNs and SP cells were strongly expressed stem cell marker genes *(ABCG2, GNL3* and *OCT4)* and the thyroid differentiation marker *(PAX8),* critical for thyroid specific gene expression indicating the proliferation of thyroid follicles. The mean C_T and standard deviation for the reference gene (ACTB) and target genes (*ABCG2*, *GNL3*, *OCT4* and *PAX8*) were calculated from the raw data (Table 4).

Groups	Genes (mean C_T)				
	ABCG	GNL3	OCT4	PAX	ACTB
Control-Adult mice	31.14	30.57	18.93	31.67	32.49
24hrs.	24.42	24.07	21.18	28.18	19.37
15 day	31.56	27.73	32.34	30.51	26.95
30 day	29.62	28.91	33.96	28.59	23.13
Old mice	30.58	29.51	12.29	28.78	24.99
OLD + THY (Se)	31.92	34.17	33.15	32.30	26.69

Table 4: *Mean threshold cycle (mean C_T) values of gene in groups were derived from experimental C_T values of samples at day-1, day-15 and day-30, 2-4 months (adult-control mice), 12-24M (old/senile mice) and old thyroid mice, treated with selenium (5mcg/day) for 20 days, against the reference (ACTB).*

PCR gene expression analysis

Evaluation of the expression pattern of *ABCG2, GNL3, OCT4* and *PAX8 genes* (Graphs 2) among five conditions of mice were done by using $\Delta\Delta C_T$ (Livak) method (Table 5). It calculated the relative expression (RE) and differential expression (Rq or FC) of genes at different treatment points. The analysis of these data revealed that the relative quantification of *OCT4 and PAX* genes via control represented upregulation only in the old age group, indicating genetic mutation at the old age especially for *OCT4*. The fold change in comparative differential expression of *OCT4* between old age group and control group was 3.07 times up- regulated in old age group with respect to control group.



Graph (2) : *Combined expression pattern of ABCG2, GNL3, OCT4, and PAX8 genes during four different age conditions at 1st day, 15th day, 30th day, 12 - 24 months (old/senile mice) and additional old thyroid mice, treated with selenium (5 mcg/day) for 20 days. 2 - 4 months adult mice were used as control, and ACTB were used as reference genes.*

Description: *Rq graph represents the comparative expression quantification of gene (test vs control) where control is normalized to 1; Rq value above 1 indicates upregulation and below 1 indicates downregulation.*

Groups	Genes (ΔC_T) ACTB			
	ABCG	GNL3	OCT4	PAX
ΔC_T Control-Adult mice	-1.35	-1.92	-13.56	-0.82
ΔC_T 24hrs.	5.05	4.70	1.82	8.81
ΔC_T 15 day	4.62	0.79	5.40	3.56
ΔC_T 30 day	6.49	5.77	10.82	5.45
ΔC_T Old mice	5.58	4.51	-12.70	3.79
ΔC_T OLD + THY (Se)	5.23	7.48	6.47	5.61

Table 5a: *ΔC_T values of each sample/group was derived from their mean C_T values of target and reference genes, ΔC_T = [C_T target gene – C_T reference gene].*

Groups Comparisons		Genes ($\Delta\Delta C_T$) ACTB			
ΔC_T Control	ΔC_T Test	ABCG	GNL3	OCT4	PAX
Control-Adult mice	24hrs.	6.41	6.63	15.38	9.63
	15 day	5.97	2.71	18.96	4.38
	30 day	7.84	7.70	24.39	6.27
	Old mice	6.94	6.43	0.86	4.61
	OLD + THY (Se)	6.59	9.41	20.03	6.43

Table 5b: *$\Delta\Delta C_T$ values as calculated by $\Delta\Delta C_T$ = [ΔC_T Test – ΔC_T Control].*

Groups Comparisons		Genes (Rq) ACTB			
ΔC_T Control	ΔC_T Test	ABCG	GNL3	OCT4	PAX
Control-Adult mice	24hrs.	0.03	0.03	6.16E-05	3.33E-03
	15 day	0.01	0.06	7.76E-07	0.02
	30 day	0.01	0.01	1.16E-07	0.03
	Old mice	0.05	0.06	3.07	0.23
	OLD + THY (Se)	2.59E-04	3.66E-05	2.32E-08	2.89E-04

Table 5c: *Relative quantification (Rq) of genes while comparing groups, $Rq = 2^{-\Delta\Delta CT}$.*

Groups Comparisons		Genes (FC) ACTB			
ΔC_T Control	ΔC_T Test	ABCG	GNL3	OCT4	PAX
Control-Adult mice	24hrs.	-32.28	-37.58	-16229.16	-300.74
	15 day	-158.18	-16.52	-1289293.87	-52.57
	30 day	-90.16	-81.60	-8650804.52	-30.35
	Old mice	-21.96	-15.50	3.07	-4.36
	OLD + THY (Se)	-3858.75	-27289.93	-43025097.39	-3456.06

Table 5d: *Fold change (FC) to represent differential expression i.e how many times gene is up or down regulated in test group with respect to control group? FC = Rq (if Rq > 1), "FC=0 (if Rq = 1), and FC = - (1/Rq) (if Rq < 1).*

Relative quantification of genes (Rq)

Combined expression pattern of *ABCG2, GNL3, OCT4* and *PAX8* genes during four different age conditions at 1st day, 15th day, 30th day, 12 - 24 months (old/senile mice) and additional old thyroid mice, treated with selenium (5 mcg/day) for 20 days. 2 - 4 months adult mice were used as control and ACTB were used as reference genes was represented in Rq graphs in (Graph 2). The relative expression of *OCT4* and PAX genes versus control represented upregulation only in the old age group, indicating genetic mutation at the old age especially for *OCT4* gene (Graph 2).

Discussion

In the present study, statistical analysis of thyroid function tests revealed that there were a significant increase in TSH, T3 and T4 levels with age and reaching their maximal levels in the adult age (G4). While there was a significant statistically decrease of thyroid function tests at the old aged group, there was a significant statistically increase of thyroid function tests at the aged animal group treated with selenium, indicating that the aged thyroid gland could restore its activity and the importance of selenium supplementation in old age.

The significance of thyroid function test assessments were in agreement with many studies as they reported that the assay of thyroid function levels were used to measure the thyroid activity, discover the thyroid diseases and follow up the supplemental therapy of hyperthyroidism [32].

Thyroid function levels were changed with the advancement of age, as previously reported [33-36]. In the aged persons, a decreased T3/T4 ratio were associated with a decrease in plasma selenium levels. Thyroid diseases, increasingly common among the general population, was also rising among the elderly [37]. Anatomical and functional changes in relation to age were detected in the aged thyroid gland. There was less conversion of inactive T4 to active T3 and decreased the up take of iodine [38,39].

In the present study, TSH level and selenium levels were decreased in the aged mice. These results were confirmed many studies as they demonstrated that aging was associated with a decrease of selenium [34,40] and TSH plasma levels [41-44].

In the present study, the treatment of the aged mice with selenium induced a marked improvement of TSH, T3 and T4 levels which reaching the levels nearly as those of the adult group.

These results were in agreement with many authors. Selenium was used as a building block of important proteins called selenoproteins which were essential for transforming T4 to T3. If the body missed selenium, production of T3 would be lowered and the thyroid gland became non-functional. The selenoproteins were essential antioxidants which removed the free radicals (ROS reactive oxygen species) that were generated during synthesis process of thyroid hormones. This was a crucial function in preventing thyroid gland damage and premature ageing [22,23].

Regarding the immunohistochemical results, at 1st day old mouse, thyroid sections stained by H&E revealed that most of the cells in the central zone of gland were organized into irregular solid masses (clusters or cords) and were surrounded by connective tissue cells, fibres and vascular sinuses, they suggested to be the solid cell nests (SCNs) as demonstrated by many studies [4,5].

Immunohistochemical expression was done by using the stem cell marker P63 according to [4,5,8]. P63 was highly expressed in developing stem cells [1,4-6,8,45]. SCNs were appeared in H&E stained sections, formed of two types of cells, the primary, dominant cells and the C cells. The primary cells, forming the most percentage of SCNs, were elongated cells with central oval nuclei with strongly eosinophilic cytoplasm. The C cells were few in number and identified by their clear cytoplasm and small central nuclei. Cystic SCNs indicating the proliferative function of SCNs were seen, they were lined by dominant primary cells and follicular epithelium with colloid in the lumen. The nuclei of the main cells only gave strong P63 expression indicating the stem cells like - characters. While the C- cells gave negative P63 expression.

These results were in agreement with results of many studies. They demonstrated that P63 expression in the SCNs cells was restricted only to the nuclei in the primary, main cells of SCNs showing stem cell like patterns [1,6,46,47].

Regarding the stem cells expression in the aged group, P63 was immune-expressed in few populations of cells and few solitary scattered cells indicating the detection of stem cells at old age but with less number. P63 expression of thyroid stem cells was expressed in many studies (8, 45 in mouse and 4 in human). In a recent study at 2019**,** they studied the expression of p63 in the lung as novel features of aging. They demonstrated that the protein expression profiles of p63, was lowered in the lungs of 14-month-old as compared with 7-9-week-old mice [48].

In the present study, thyroid gland of aged mouse treated with selenium showing an apparent improvement of the thyroid structure as many follicles restored their shapes and their lumina showed vacuolated colloid indicating the restoring of follicular activity and colloid absorption. The follicular cells became cuboidal in shape with spherical deeply stained nuclei. P63 expression were more than those of the old age and was very similar to those of the adult age, indicating the activation of the silent stem cells and the beginning of their proliferative activity.

These results were in agreement with many studies. The prophylactic effect of antioxidant treatment in acute liver failure by enhancing the anti-stress ability and therapeutic efficacy of the human mesenchymal stem cell was demonstrated [49]. The antioxidants, selenium and vitamin E supplementation decreased the peroxidation process and could increase the T3 and T4 concentration in plasma [50,51]. Selenium had anti-oxidative, anti-inflammatory, anti-neoplastic and anti-aging properties [23,26,52].

In the present study, the gene expression pattern of *ABCG, GNL3, OCT4*, and *PAX8* were evaluated by RT-qPCR analysis for e thyroid stem cell populations and thyroid differentiation cells on the mouse at different ages and determined the effect of selenium on the expression pattern. It revealed that the thyroid stem population cells were highly expressed the stem cell marker genes *ABCG2, GNL3 and OCT4 and* the thyroid differentiation marker *PAX8.*

Stem cell markers were genes and their protein products used by scientists to isolate and identify stem cells. The thyroid stem cells were genetically expressed by using RT-qPCR for expression of stem cell marker, *ABCG gene* [4,8,11], stem cell marker, *OCT4 gene* [7], *GNL3 protein* [8,15] and gene associated with thyroid differentiation, paired transcription factor *Pax 8 gene* [18,53].

The detection of adult stem cells in human thyroid glands as populations and expression of all stem cell markers including *OCT4 and of PAX8* was studied. RT-qPCR, flow cytometry, and immunofluorescence were used to detect the expression of the stem cell marker *OCT4* and. Their findings suggested the presence of adult stem in the human thyroid gland [7]. Quantitative RT-PCR analysis for gene expression in SP cells in the mouse thyroid showed stem cell-like characters The SP cells were strongly expressed *ABCG2, GNL3 and OCT4,* whereas the expression of genes encoding the thyroid differentiation markers, thyroglobulin (Tg) and paired *PAX8*, critical for thyroid specific gene expression, are low in SP cells as compared with the main population cells [8].

RT-qPCR analysis revealed that *ABCG2* was up-regulated in thyroid SP cells, which ensured the criteria for SP cells; their phenotype was attributed to the function of *ABCG2* [8].

PAX8, thyroid differentiation transcription factor [18,19] this nuclear protein was involved in thyroid follicular cell development and expression of thyroid-specific genes. *PAX8* and other transcription factors played a role in binding to DNA and were regulating the genes that derived thyroid hormone synthesis (Tg, and TSHr). Mutations in *PAX8* gene had been associated with thyroid dysgenesis, thyroid follicular carcinomas and atypical follicular thyroid adenomas [19]. Side population cell-derived thyroid cell line cells had the capacity to differentiate into thyroid to a limited degree. Two-step process of thyroid folliculogenesis (NKX2 and *PAX8*). Thyroid follicle formation required NKX2-1 expression, while the expression of *PAX8* was required for the follicle to become functional, resulting in the expression of many thyroid differentiation marker genes and eventual thyroid hormone synthesis [54].

In the present study, the relative quantification of *OCT4* and PAX genes via control represented upregulation only in the aged group, indicating genetic mutation at the old age especially for *OCT4 gene*.

This results confirmed the results of many studies. the downregulation was the process by which a cell decreased the quantity of a cellular component, such as RNA or protein, in response to an external stimulus. The complementary process that led to the increase of such components was called upregulation. Downregulation or upregulation of an RNA or protein might also increase by an epigenetic alteration, this occurred, during aging, during drug addiction or progression to cancer [55,57].

In the present study, expression of *ABCG2* and *GNL3* was detected in the aged group and appeared like those of the control group. Gene expression of *OCT4* was greatly decreased in old age; this might be as *OCT4* is a stem marker for embryonic and adult cells only. Also, the gene expression of *PAX8* was moderately decreased in old age indicating the decreased activity of the thyroid follicles.

In the old age, the amount of stem cells and their renewal ability were decreased. There were many predisposing factors for cellular senescence, apoptosis, autophagy, and oxidative stress, which finally accumulated and led to aging [58-61].

In the present study, the fold change in comparative differential expression of *OCT4* between the aged group and control group was 3.07 times up- regulated in old age group with respect to control group. The fold changes were expressed only in the aging group indicating that their gene expression alterations.

The changes in gene expression in the old age showed gene expression alterations [56]. In each organ, most of genes altered significantly during aging with upregulation than downregulation. In old age, The expression profiles in aging organs and the fold-changes were generally consistent with similar microarray studies [55,61-63]. The decreased renewal ability of stem cells in old age raised questions as to whether the donor age should be put in consideration during transplantation with stem to ensure the largest possible number of viable, functional, and non-deteriorated stem cells. Although stem cells was expressed into old age, they were non functional as lost their regenerative ability [64].

In the present study, gene expression in the selenium treated old group gave nearly the same expression as those of the adult and 30 days old group, indicating the improvement and restoration of the gene expression pattern as the younger age.

The anti-aging therapies aimed to use the power of stem cells for growth and regeneration by activating the endogenous stem cells or increased their number and functions [58,61].

In the present study, RT-qPCR data analysis on the thyroid mouse of old age treated with selenium was supported the immunohistochemical results as the gene expression pattern of *ABCG, GNL3, OCT4*, and *PAX8* for the thyroid stem cell populations and thyroid differentiation cells showed improvement of their expression pattern which became nearly very similar to those of the adult group.

This result indicates the ameliorative effect of selenium on enhancing the aged stem cells ability to regenerate and repair the aged thyroid tissue and confirming the recommendation by the US Food and Nutrition Board's Committee on Dietary Allowances which had been reported that selenium was an essential trace mineral for humans because of its antioxidant and anti-inflammatory properties and proposed a recommended daily intake of 50 - 200 micrograms/day [52].

Conclusion

From the present study, it could be concluded that the stem cells have been expressed in the mouse thyroid tissues at all ages and the prophylactic role of selenium to ameliorative the aged stem cells had been detected.

Recommendations

The daily supplementation of selenium is highly recommended in old age for activation of aged thyroid stem cells, protection from physiological senile hypothyroidism and improvement of the thyroid function.

Acknowledgement

The authors of the manuscript are very grateful to the staff of King Fahd Medical Research Center, King Abdulaziz University, Jeddah, KSA for their technical assistance and support. Great thanks for the staff of GenaTi Unit at King Fahd Medical Research Centre, KAU, Jeddah, Saudi Arabia.

Disclosure Statement

The KFMRC had no role in study design, data collection and analysis, the decision to publish or preparation of the manuscript.

Conflicts of Interest

The authors have declared that no conflicts of interest exist.

Funding Sources

Faculty of Science, King Abdulaziz University, Saudi Arabia, Jeddah.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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