The Therapeutic Potential of Stem Cells-Conditioned Media on Histologic Changes of Immune Checkpoints Inhibitor Pembrolizumab on Rats Thyroid Gland

Original Article

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ABSTRACT

Background: Immunological checkpoints inhibitor (ICI) Pembrolizumab (PEMB) is a monoclonal antibody that induces a potent antitumor immune response. Hypothyroidism and adrenal insufficiency have been associated with its addition to the therapy protocol. Conditioned media (CM) is a culture media for stem cells comprising soluble proteins, lipids, nucleic acids, and extracellular vesicles (EV). Aim of the Work: This research aimed to determine whether mesenchymal stem cells conditioned media (MSCs-CM) could ameliorate thyroid gland histological disruptions caused by pembrolizumab.

Materials and Methods: 45 adult male rats were incorporated in this work and classified into: control group (I), Pembrolizumab treated group (II): Pembrolizumab was administered intraperitoneally (i.p.) at a dose of 3 mg/kg three times a week for four weeks, and conditioned media treated group (III): was treated as group II and then received 200µl of MSCs-CM via tail vein twice a week for three weeks. We measured thyroid hormones and oxidative markers. The thyroid gland was examined by Light and electron microscopy. Statistics were used to analyze all the data.

Results: PEMB group shows desquamation of follicular cells into the lumen, cytoplasmic vacuolations and wide interstitial spaces among follicles. Some follicles appear involuted, others appear dilated and some follicles lack colloid. MSCs-CM group shows improvement in the thyroid sections' structural appearance. The majority of the follicles are colloid-filled. Cuboidal cells having large, rounded central nuclei lined the majority of thyroid follicles. Some follicles still have vacuolated cytoplasm.

Conclusions: MSCs-CM is found to play a therapeutic role to improve PEMB thyroid ultrastructure histological alterations.

Key Words: Mesenchymal stem cells conditioned media (MSCs-CM), Pembrolizumab (PEMB) and Thyroid gland.

Revised: 18 September 2023, **Accepted:** 19 September 2023

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ISSN: 1110-0559, Vol.6, No.2

INTRODUCTION

The immunotherapeutic agent pembrolizumab is a humanized monoclonal IgG4 and it is one of the immune checkpoint inhibitors (ICIs)[1]. The immune checkpoints are responsible for regulating the immune system and preventing autoimmunity. They are proteins presented on the cell membranes of cytotoxic T cells. These proteins include cytotoxic T-lymphocyte-associated-4 (CTLA-4) and programmed cell death protein 1 (PD-1)[2].

They inhibit T cells' immune activity and maintain self-tolerance by binding to inhibitory ligands (PD-L1) and (PD-L2). Checkpoint proteins can also be found on B cells, natural killer cells, macrophages and dendritic cells[3].

Tumor cells express PD-L1; therefore, they can escape immune attack by suppressing the antitumor T cell action. The immune checkpoint inhibitor pembrolizumab blocks PD-1 receptors (CD279). Therefore, release the inhibition on T-cell activity through preventing T-cell PD-1/tumor cell PD-L1 interaction and restoring the enhanced antitumor immune-mediated response[4 - 5]. Pembrolizumab has been approved to treat different cancer types since September 2014 and has become antitumor immunotherapy[6].

Some inflammatory diseases, such as chronic lymphocytic thyroiditis (CLT) and Hashimoto's thyroiditis (HT), have also been found to express PD-L1[7].

Although the survival rate of immune therapy drugs is higher than other chemotherapy, their adverse autoimmune endocrinal complications are also high and the proposed mechanism that stands behind it is still not well known[8].

The thyroid and pituitary glands are pembrolizumab's most commonly affected organs[9]. Tan *et al.* (2019)[10] mentioned that

 DOI: 10.21608/jmh.2023.237352.1120

hypothyroidism occurs in nearly 68 % of patients treated with pembrolizumab. It is suggested to be owed to antibody-dependent phagocytosis or cell-mediated cytotoxicity, complement-dependent cytotoxicity and release of inflammatory mediators. Jabkowski *et al*. (2021)[11] added that if a hidden autoimmune disease preexists in a patient before immune therapy treatment, it would be a high-risk factor for evolving severe complications.

Myocarditis and rheumatological complications were recorded, especially in female patients[12]. Diabetes mellitus and diabetic ketoacidosis[13] and vasculitis[14] are also common serious complications. Moreover, pembrolizumab can cause abortion and/or stillbirths if taken by a pregnant female having cancer[15].

As manifestations of complications do not appear in most cases until reaching the terminal stage[16]; so, paying attention to these complications from the beginning of immune therapy is necessary by frequent routine investigations for all hormonal profiles and blood glucose levels[8]. It is worth trying to prevent or control these complications using supportive agents such as mesenchymal stem cells conditioned media (MSCs-CM). With the emergence of stem cell therapy, MSCs-CM has been explored as a thrilling solution for many diseases. They contain secretomes, microvesicles, or exosomes secreted in the culture media of stem cells. They are now widely used and transported as drugs, however; they need recurrent applications[17 - 18].

Owing to the limited survival of MSCs, the improved tissue structure and functions point to the paracrine effect from these growth factors in the culture media[19].

There are conflicts between the benefits and hazards of pembrolizumab usage. In addition to the scientific debate among researchers about the exact etiology and pathogenesis of pembrolizumab affecting the thyroid gland in cancer patients, as for our knowledge, no or little previous studies investigated the experimental effect of pembrolizumab on the thyroid gland in rats. So, we aimed to examine the impact of pembrolizumab on the thyroid gland structure and function in male adult rats and to explore the probable role of mesenchymal stem cells conditioned media (MSCs-CM).

MATERIALS AND METHODS

Animals:

Forty-five healthy male adult albino rats (with an average age of 14 - 18 weeks and body weight ranging from 180 - 200 grams) were utilized. They were bought and kept at the Breeding Animal House, Faculty of Medicine, Zagazig University. Rats had free access to water and food. They housed in plastic cages with controlling illumination and temperature. They were kept for a one-week acclimatization period. All the experimental protocols were approved by the Medical Research Ethics Committee of the Faculty of Medicine, Zagazig University, Egypt (ZU-IACUC/3/F/1272023/). We followed the National Institutes of Health Guide for the Care and Use of laboratory animals (NIH Publications "No.8023, revised 1996").

Chemicals and preparation:

Pembrolizumab: pembrolizumab (KEYTRUDA) is available from Schering-Plough Labo NV, Belgium, as 50 mg powder diluted in 2 ml of distilled water. Pembrolizumab was freshly prepared before injection.

Preparation of MSCs-CM: To generate MSCs-CM, cells were allowed to grow to 80 - 90 % confluence (approximately 3×10^6 MSCs per 55-cm^2 dish) (this take nearly from 10 : 15 days) then the MSCs were detached and subcultured into new cell flasks. The optimal passage used containing maximum amount of growth factors is the 3^{rd} : 4^{th} passage. The cells washed thoroughly and cultured in 10 mL serum-free α-MEM. The conditioned media was collected 24 hours later. The conditioned growth media was concentrated 25-fold through ultrafiltration units (Millipore, Bedford, MA)[20 - 21].

The Preparation of MSCs-CM was done at the Biochemistry Department, Faculty of Medicine, Zagazig University.

Experimental protocol:

Rats were classified into three main groups:

Group I (control group): Includes 27 animals that were equally divided into three subgroups (9 rats each),

- Subgroup1a (Negative control group): Nine rats were fed daily on a standard diet for four weeks and received no treatment.
- Subgroup 1b: Included nine rats. The animals of this group received a daily dose of 2 ml of distilled water injected intraperitoneal (i.p) for four weeks as a vehicle of Pembrolizumab[22].
- Subgroup 1c: included nine rats. This group were injected with 200 µl MSCs-CM through

the tail vein for each rat[20] twice per week for three weeks[21].

Group II (Pembrolizumab treated group): Included nine rats that were injected with freshly prepared Pembrolizumab 3mg/kg intraperitoneal (i.p) injection, three times per week for four weeks[22].

Group III (Pembrolizumab + Mesenchymal Stem Cells-Conditioned Media): Included nine rats that were treated with freshly prepared Pembrolizumab as group II and then injected with 200µl MSCs-CM via tail vein for each[20] twice per week for three weeks[21].

Biochemical analysis:

Before scarification, retro-orbital blood samples were collected in capillary tubes. Serum total T3, total T4 and TSH levels were tested using ELISA test kits (My Biosource kits) with CAT. NO. MBS2000350, MBS580037 and MBS729687 respectively.

Light and immunohistochemical study:

The thyroid gland from each animal was carefully dissected and the specimens were immediately immersed in formol saline 10 % for 48 h to be processed to get 5µm-thick paraffin sections and stained with hematoxylin and eosin (H&E) to display the histological details[23].

Immunohistochemical staining was made for the localization of CD8+ (C8144/B; DAKO, Gloustrup, Denmark) diluted 1:100 in PBS. CD8+ is a transmembrane glycoprotein co-receptor of T-cell receptor (TCR) to detect the inflammatory cells in the stroma between the follicles.

Calcitonin immunoreactive C cells (DAKO A-576; Dako, Glostrup, Denmark).

Avidin-biotin–peroxidase complex method was performed. Processing the paraffin slices for immunostaining was achieved. Endogenous peroxidase was detached. A nonspecific attachment was blocked. Sections were covered with the primary and secondary antibodies (biotinylated) and labeled horseradish peroxidase was added to sections. Staining at the antigen place by DAB appears brown[24 - 25].

Electron microscopic study:

Thyroid tissue was fixed for 1 hour in phosphate buffer with 1 % glutaraldehyde and 4 % paraformaldehyde. Sections were post-fixed in osmium tetroxide, trimmed, dehydrated and epoxy resin embedded. Uranyl acetate and lead citrate were used to stain ultrathin slices (80 – 90 nm). A Jeol electron microscope was utilized to examine the tissues. Tanta University Faculty of Medicine's Unit of Electron Microscopy performed the TEM processing (Jeol JEM – 100SX ELECTRON MICROSCOPE)[26]. The magnifications of the E/M figures have been explained according to the illustration bar present at the bottom part of the figures.

Morphometric study:

Ten non-overlapping areas from each section of each animal group were subjected to semiquantitative microscopical analysis at 400 magnifications.

The area percentage of CD8+ and C cells calcitonin immunoreactivity was measured. Measures were all obtained at 400 magnifications.

Statistical analysis:

The data from the image analyzer were analyzed using one-way analysis of variance (ANOVA) to compare between groups (more than two groups). ANOVA test was statistically significant when the P value < 0.05[27].

RESULTS

Biochemical results:

There was a highly significant drop in total T3 and total T4 levels when comparing pembrolizumab-treated rats (group II) with group I and group III (Pembrolizumab + MSCs-CM) ($p < 0.001$). There was a Non-significant difference when relating group III to group I ($p > 0.05$). Regarding TSH level, there was a highly significant elevation when comparing pembrolizumab-treated rats (group II) with group I ($p < 0.001$) and group III (Pembrolizumab + MSCs-CM) ($p < 0.001$). There was a Non-significant difference when relating group III to group I ($p > 0.05$) (Table 1).

Table 1: Comparison between serum total T3 in µg/dl, serum total T4 in µg/dl and serum TSH in µlU/ml of different groups:

Groups	Group I (control group)	Group II (pembrolizumab-treated group)	Group III (Mesenchymal stem cells conditioned media group)
Total T3	39.2 ± 1.1	19.9 ± 0.11[a,b]	38.1 ± 0.65[NS]
Total T4	4.06 ± 0.32	2.77 ± 34[a,b]	3.80 ± 38[NS]
TSH	0.126 ± 0.009	0.281 ± 0.053[a,b]	0.134 ± 0.024[NS]

Values are expressed as mean± standard deviation (SD).
a: Highly significant difference relating group II to the control group ($p < 0.001$).
b: Highly significant difference relating group II to group III ($p < 0.001$).
NS: Non-significant difference relating group III with group I ($p > 0.05$).

Histological results:

Data collected from subgroups (Ia, Ib and Ic) regarding light and electron microscopy results were nearly similar; hence subgroup (Ia) data were chosen for comparison with other groups.

H&E stained results:

Examination of H&E stained sections of the control group (Group I), they showed that thyroid follicles were lined by cuboidal cells having rounded central nuclei. Homogenous colloid filled the lumina of the follicles. The parafollicular cells were small and oval. In between follicles, interfollicular cell groups could be detected (Figure 1a). Pembrolizumab-treated rats (Group II) displayed disturbance of thyroid tissue as numerous disorganized, damaged follicles. Many follicles were lined by numerous layers of follicular cells and desquamation of follicular cells into the lumen were seen. Flat cells and cuboidal cells having rounded nuclei were seen lining the follicles. The cytoplasm of many follicular cells was vacuolated. Moreover,some follicles had large interstitial spaces between them. Some involuted follicles were seen beside other dilated ones. In addition, some follicles lacked colloid and others had an abundant amount (Figure 1b, c). Many follicles were lined by numerous layers of follicular cells still seen with engorged blood vessels (Figure 1d). Group III (Pembrolizumab + MSCs-CM) showed improvement in the thyroid sections' structural appearance relative to the pembrolizumab-treated group. The majority of the follicles were colloid-filled. Cuboidal cells having large, rounded central nuclei lined the majority of thyroid follicles. Some follicles still had vacuolated cytoplasm (Figure 1e).

2- Immunohistochemical results

CD8+ immunostained sections showed few brown positive membranous immunoreactions seen in the stroma between the follicles of the control group (Figure 2a). More positive reaction appeared in the pembrolizumab treated group (Figure 2b). MSCs-CM treated group revealed diminished reaction (Figure 2c).

For calcitonin C cells immunoreaction, brown cytoplasmic reaction was seen in the control group parafollicular cells. The follicular cells showed no reaction (Figure 3a). Pembrolizumab treated group revealed more reaction (Figure 3b). MSCs-CM treated group revealed few reactions (Figure 3c).

3- Electron microscope results

Control rats showed follicular cells containing oval euchromatic nuclei, rough endoplasmic reticulum and many mitochondria. Follicles were bounded by a thin basement membrane and contained homogenous colloid (Figure 4 a). Junctional complexes were seen between cells (Figure 4 b). Parafollicular cells had large, central nuclei and many electron-dense secretory granules (Figure 4 c).

Pembrolizumab-treated rats showed follicular cells containing irregularly shaped nuclei. Cytoplasmic vacuolations, lysosomes, rough endoplasmic reticulum and destructed junctional complexes (Figure 5a, b, c, d). other follicular cells attributed shrunken irregular nuclei and swollen mitochondria. Engorged blood capillaries were seen (Figure 5e). Additionally, some cells appeared separated with macrophages in between. The macrophage had abundant lysosomes and many pseudopodia (Figure 5f). Also, some mast cells, with their characteristic cytoplasmic granules and many pseudopodia, were identified (Figure 5g).

Para follicular c-cells appeared with large and central nuclei. Their cytoplasm had many electron-dense secretory granules (Figure 5h).

MSCs-CM treated group displayed follicular cells containing rounded euchromatic nuclei, rough endoplasmic reticulum and lysosomes. Apical microvilli were seen (Figure 6a). Two layers of cells aligned some follicles. Dilated vasculatures and intact apical microvilli were obvious (Figure 6b). Para follicular cells had large and central nuclei. Their cytoplasm had many electron-dense secretory granules (Figure 6c).

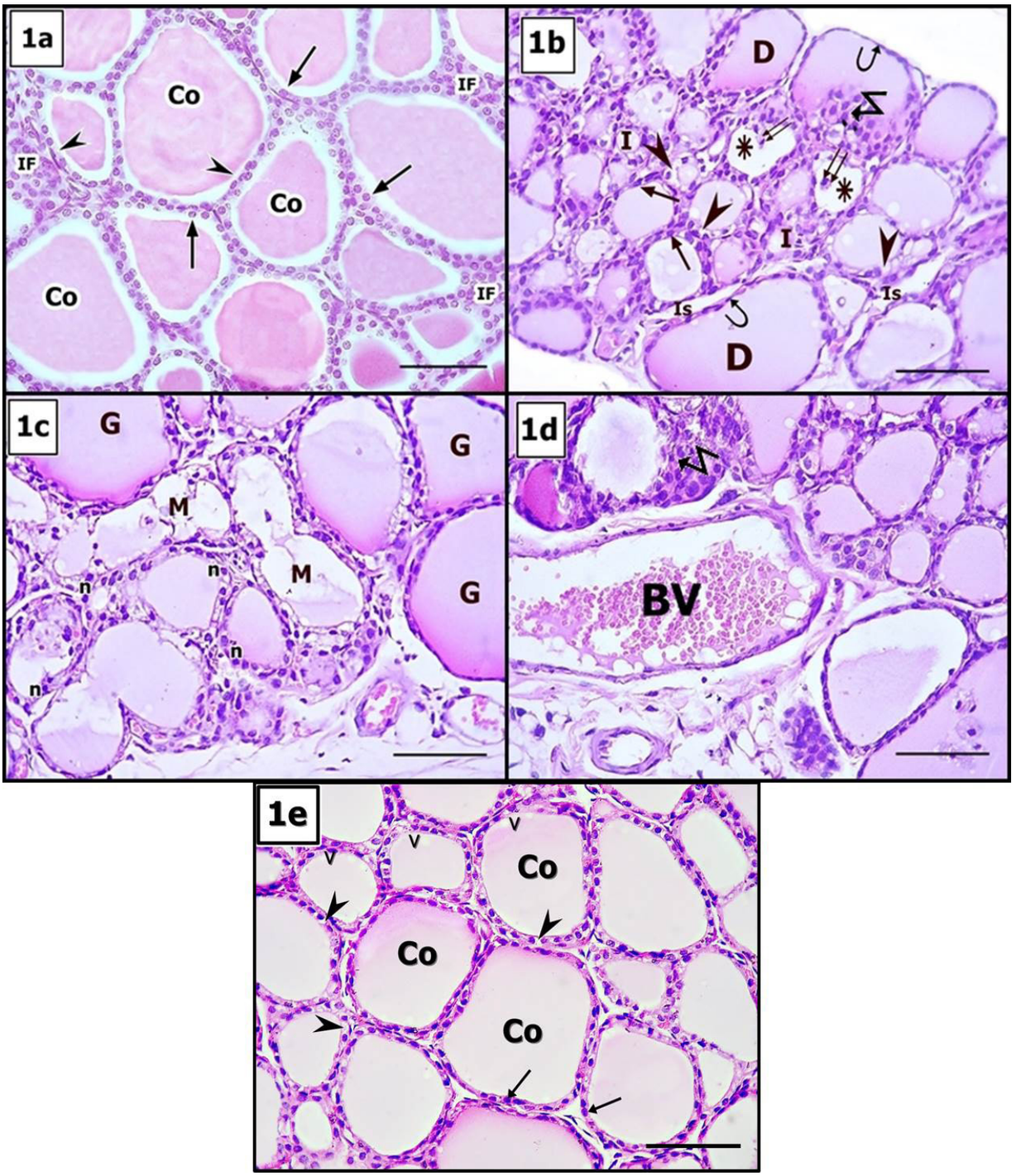


Figure 1: Photomicrographs of H&E-stained slides of the thyroid gland. (a): control group displays thyroid follicles aligned by cuboidal cells with rounded central nuclei (arrows). Homogenous acidophilic colloid (Co) fills the lumina of the follicles. Small, oval, parafollicular cells (arrowheads) and interfollicular cell groups (IF) can be detected. (b-d) Pembrolizumab treated rats (b): many follicles were lined by numerous layers of follicular cells (zigzag arrow) and the desquamation of follicular cells into the lumen (doubled arrows). Flat cells (curved arrows) and cuboidal cells having rounded nuclei line the follicles (arrows). Cytoplasmic vacuolations (arrowheads). Wide interstitial spaces among follicles (Is). Some follicles appear involuted (I), others appear dilated (D). Some follicles lack colloid (asterisk). (c): scanty colloid (M) in number of follicles and abundant amount in others (G). Deeply stained nuclei (n). (d): many follicles were lined by numerous layers of follicular cells (zigzag arrow) are still detected with wide engorged blood vessel (BV). (e): MSCs-CM group shows colloid-filled follicles (Co) having peripheral vacuolations (v). follicles are aligned by cuboidal cells having rounded nuclei (arrows). Some follicles still have cytoplasmic vacuolations (arrowheads) (Scale Bar X40µm, H,E X 400).

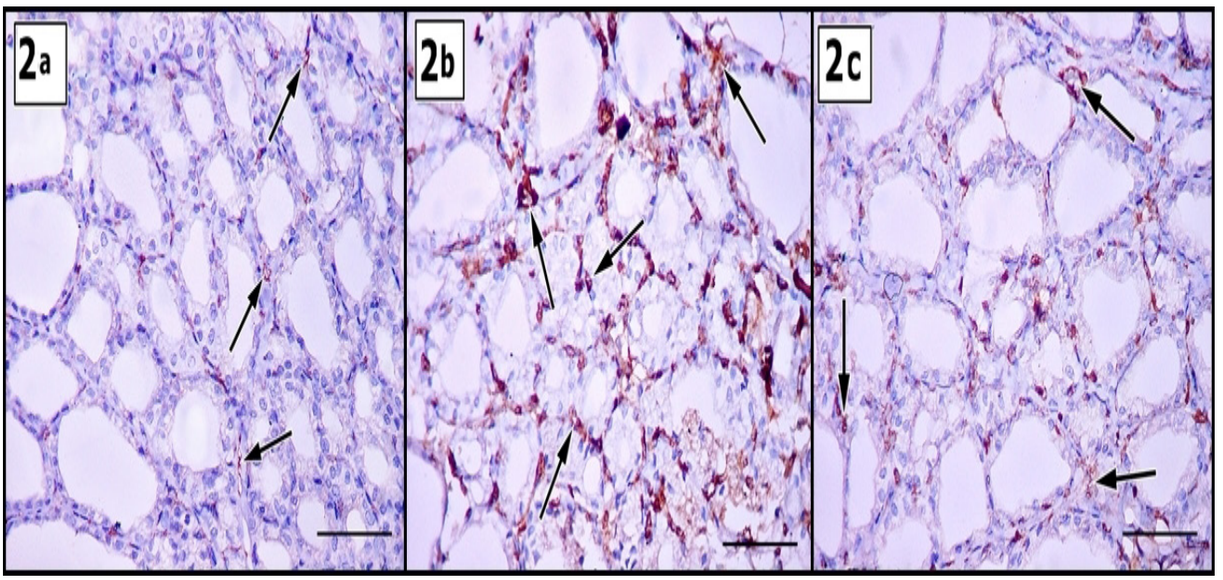


Figure 2: Photomicrographs of CD8+ immunostained slides from the thyroid glands (a): control group reveals few positive brownish membranous reactions (arrows). (b): Pembrolizumab treated group shows more positive reaction (arrows). (c): MSCs-CM treated group reveals diminished reaction (arrows) (Scale Bar X40µm,CD8+ Immunohistochemical X 400).

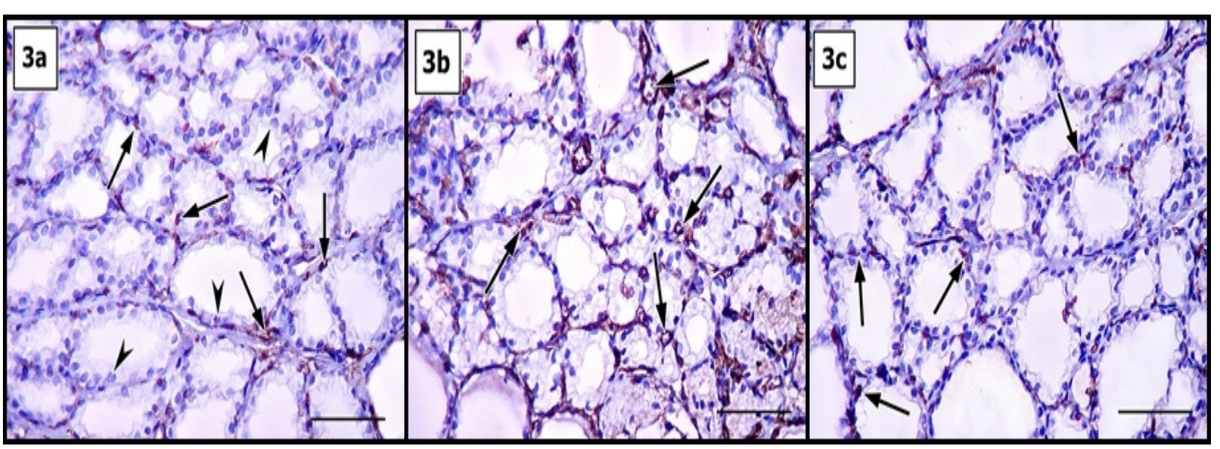


Figure 3: Calcitonin C cells immunoreaction: (a): control group reveals few positive brown cytoplasmic reactions (arrows. The follicular cells express no reaction (arrowheads) (b): Pembrolizumab treated group revealed more positive reaction (arrows). (c): MSCs-CM treated group reveals few cytoplasmic reactions (arrows) (Scale Bar X40µm,C cells immunoreaction X 400).

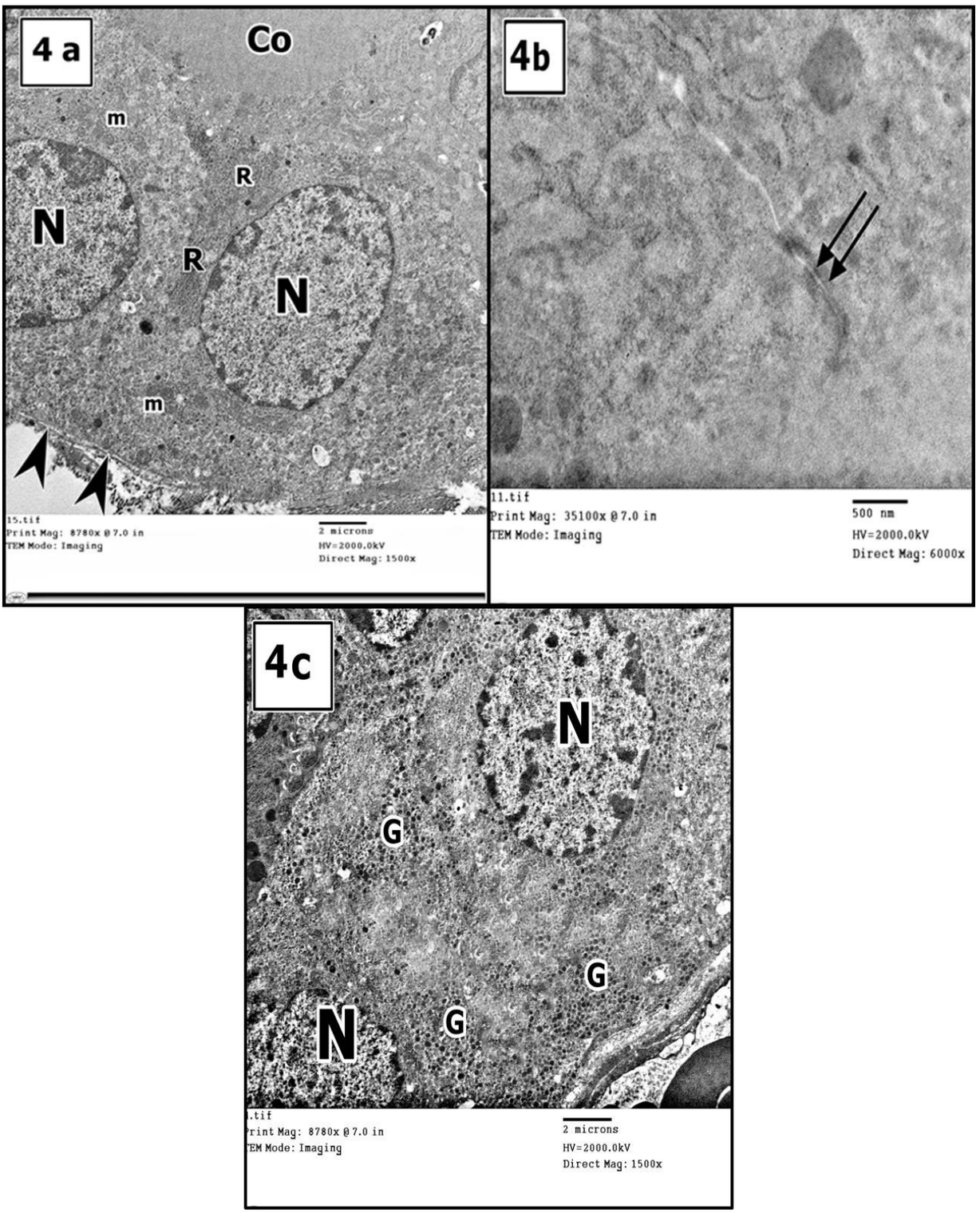
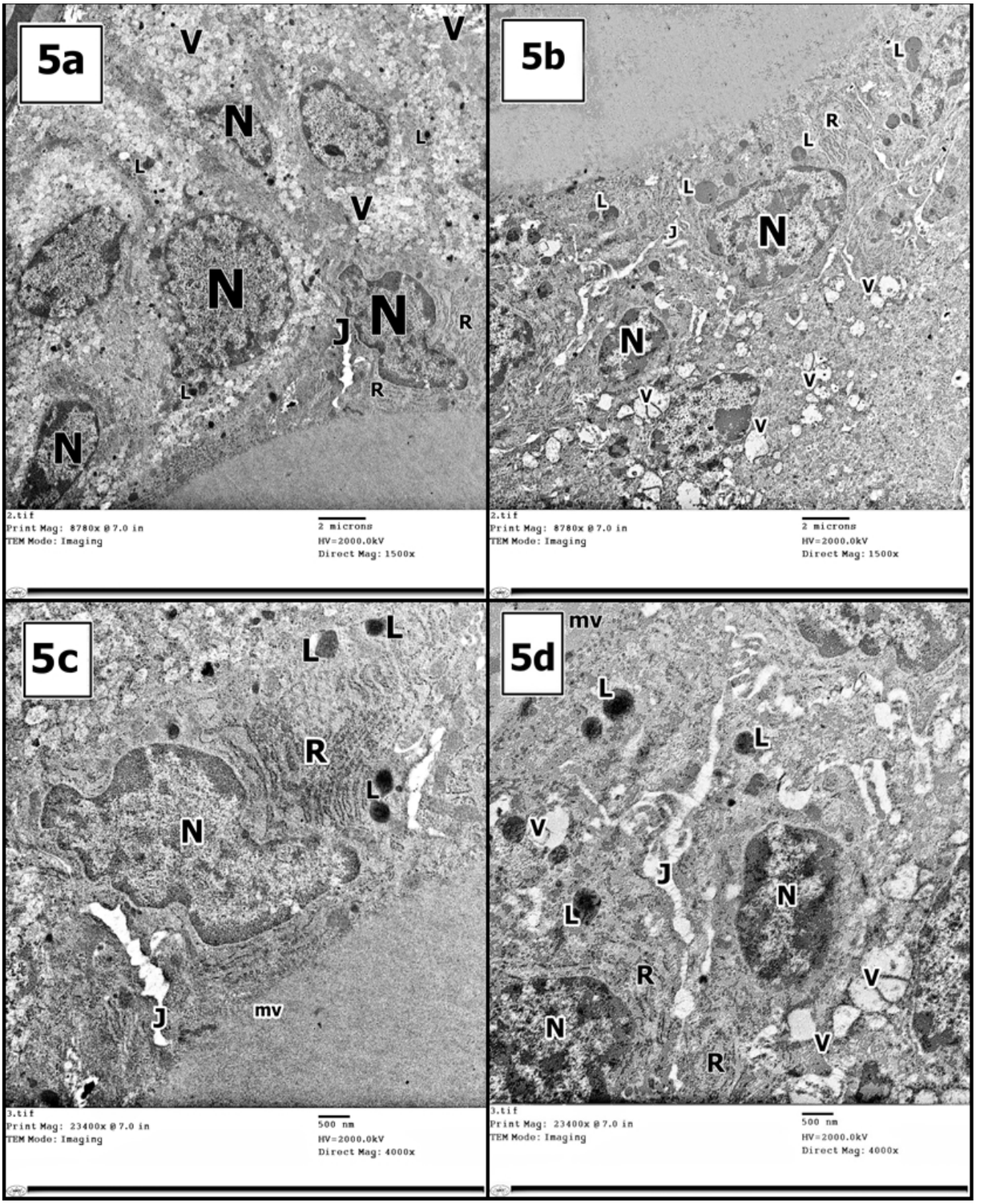


Figure 4: Electron micrograph of control group (a): show follicular cells containing oval euchromatic nuclei (N), rough endoplasmic reticulum (R), mitochondria (m). Follicles are bounded by thin basal lamina (arrowheads) and contain homogenous colloid (Co) (Direct Mag X 1500). (b): Junctional complexes are seen between the cells (doubled arrow) (Direct Mag X 6000). (c): Parafollicular cells have large and central nuclei (N) and many electron-dense secretory granules (G) (Direct Mag X 1500).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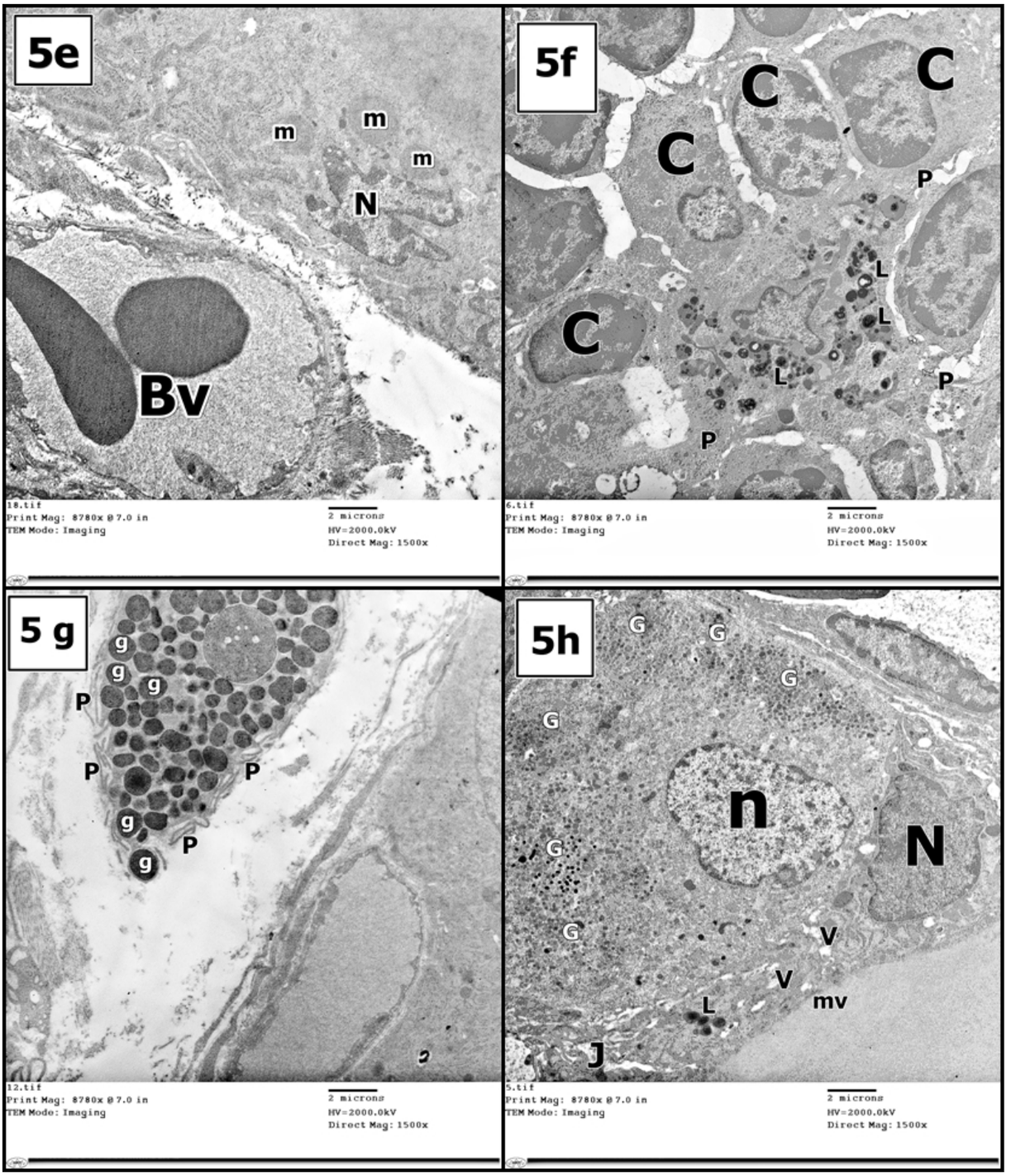

Figure 5: Pembrolizumab-treated rats showing (a, b): follicular cells containing irregular nuclei (N). cytoplasmic vacuolations (V), lysosomes (L), rough endoplasmic reticulum (R) and destructed junctional complexes (J). (Direct Mag X 1500). (c, d): Higher magnification shows irregular nuclei (N) and apical atrophic microvilli (mv). The cytoplasm holds lysosomes (L), widened rough endoplasmic reticulum (R) and destructed junctional complexes (J) (Direct Mag X 4000). (e): Follicular cell with shrunken irregular nuclei (N), swollen mitochondria (m) and engorged blood capillary (BV) (Direct Mag X 1500). (f): Some cells appear separated (C) with macrophage in-between. The macrophage has abundant lysosomes (L) and many pseudopodia (p) (Direct Mag X 1500). (g) Mast cell has cytoplasmic granules (g) and many pseudopodia (p) (Direct Mag X 1500). (h) A para follicular cell has large, central nuclei (n). Its cytoplasm has many electron-dense granules (G). The adjacent follicular cell reveals irregular nuclei (N) with atrophied microvilli (mv), disrupted Junctional complexes (J), vacuolations (V) and multiple lysosomes (L) (Direct Mag X 1500).

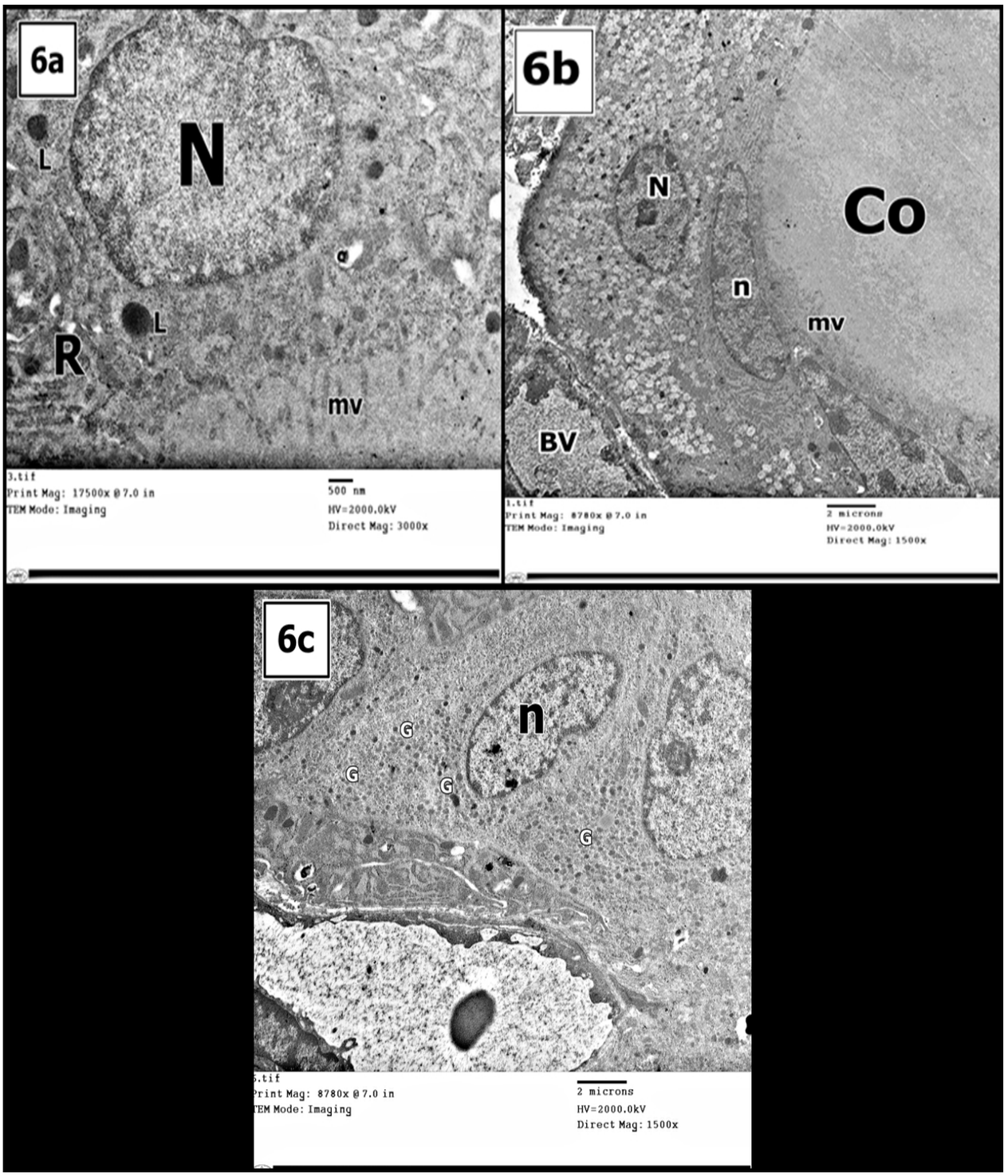

Figure 6: Electron micrograph of MSCs-CM showing (a): A follicular cells containing rounded euchromatic nucleus (N), lysosomes (L), rough endoplasmic reticulum (R) and apical microvilli (mv) (Direct Mag X 3000). (b): Thyroid follicle lined by two cell layers. A cell in one layer has a rounded nucleus (N) and a cell in the other layer exhibits a flat nucleus (n). homogenous colloid (Co) is obvious. Notice: dilated blood vessel (BV) and intact apical microvilli (mv) (Direct Mag X 1500). (c): Para follicular cells contain large and central nuclei (n). Their cytoplasm have many electron dense granules (G) (Direct Mag X 1500).

Morphometric and statistical results:

Statistical analysis of CD8+ and calcitonin immunoreactive C cells area percent showed highly significant elevation relating group II with groups I and III ($p < 0.001$). There was a Non-significant difference when relating group III to group I ($p > 0.05$) (Table 2).

Table 2: Comparison between the mean areas % of CD8+ and calcitonin immunoreactive C cells in different groups:

Groups	Group I (control group)	Group II (pembrolizumab-treated group)	Group III (Mesenchymal stem cells conditioned media group)
CD8+	21.2 ± 0.63	37.0 ± 0.73[a,b]	21.1 ± 0.82[NS]
calcitonin immunoreactive C cells	5.92 ± 0.32	16.09 ± 0.74[a,b]	6.41 ± 0.25[NS]

Values are stated as mean± standard deviation (SD).
a: Highly significant difference relating group II with the control group ($p < 0.001$).
b: Highly significant difference relating group II with group III ($p < 0.001$).
NS: Non- significant difference relating group III with group I ($p > 0.05$).

DISCUSSION

This work showed that treating rats with pembrolizumab disrupted thyroid tissue in the form of many disorganized, destroyed follicles with desquamation of follicular cells into lumina. Some empty follicles appeared combined with wide interstitial spaces. Moreover, A number of follicular cells were replaced by inflammatory cells. Similar results were perceived in a case report after seven months of pembrolizumab treatment in which there was more than two-thirds destruction of the thyroid tissues and a three-fold elevation of thyroglobulin concentration. Moreover, the thyroid Peroxisome Antibody (TPOAb) level was elevated[28].

In this work, we demonstrated microfollicles with minimal thyroglobulin and other follicles were engorged with thyroglobulin. These results are in line with[11], who found PDL1 expression in these microfollicles between clusters of CD4+, CD8+ T lymphocytes and histiocytes. PD-L1 in these follicles acted as a safeguard against thyroid follicles' lymphocyte attack. However, after treatment with pembrolizumab which inhibits the interaction between PD-1 and PD-L1, helps T cell involvement in the gland which negatively affects the gland's structure and function.

Thyroid affection from pembrolizumab passes in stages; it begins with destructive thyroiditis and hyperthyroidism, then develop hypothyroidism within 3 to 6 weeks[29]. Destructive thyroiditis and hyperthyroidism are caused by attacking thyroid follicles by the activated cytotoxic T cells releasing high amounts of T4 and T3 from the destroyed follicles until depletion of stores; it then proceeds to hypothyroidism[3 - 7]. In addition, pembrolizumab cause destructive thyroiditis by stimulating other immune and inflammatory cells, such as CD45+CD16+ natural killer cells and CD14+CD16+ monocytes[30].

We recorded inflammatory cells in the stroma between the follicles, which appeared immunohistochemically as brown positive membranous immunoreaction of CD8+ T cells and confirmed by a significant increase in their area percent in the pembrolizumab-treated group. CD8+ area percent showed a highly significant elevation when relating group II to group I ($p < 0.001$) and a highly significant increase when relating group II to group III ($p < 0.001$). There was a non-significant difference when comparing group III with group I ($p > 0.05$). CD8+ immunoreaction is also seen in the report of[31] with colloid granulomas. They mentioned that lymphocytes invade the whole thyroid. Plitnick *et al.* (2020)[32] mentioned that PD-1 with PDL-1 activates tyrosine phosphatase which in turn dephosphorylates kinases responsible for T-cell activation. Therefore, pembrolizumab restores T- cells tissue recruitment by blocking this binding.

Additionally, calcitonin immunoreactive C cells area percent showed highly significant elevation relating group II with groups I and III ($p < 0.001$). There was a Non-significant difference relating group III with group I ($p > 0.05$). High TSH values may be the cause of hyperplasia and hypertrophy of C cells as a compensatory mechanism. Martín-Lacave *et al.* (2009)[25] related C cell activities with thyroid function and reported that large C cells were abundant singly or in groups in hyperactive thyroids.

Other types of T cell activation as CD3 and CD4, are seen in thyroid tissue by Jabkowski *et al.* (2021) [11]. They also found CD20 B-cells, CD138 plasma cells and epitheloid cell granulomas in few amounts. However, CD68 histiocytes were seen in numerous amounts.

Natural killer cells are also increased[30]. Angell *et al.* (2018)[33] mentioned that extensive lymphocytic infiltration and necrotic cells invaded the whole gland up to the level that follicular cells cannot be distinguished. They added that monocytes are also activated due to increased expression of HLA-DR on their surfaces, enabling them to distinguish follicular cells with the same antigens of tumor cells as foreign cells and attack them.

Thyroid inflammatory disorders occurring with PD-1 block have been found to secrete interferon-gamma (IFNg) that elevate PD-L1 expression exposing these patients to more thyroid follicle destruction and entering a vicious cycle of deterioration[34, 7, 35 - 36].

Das *et al.* (2018)[37] suggested that if a low inflammation level in a certain organ precedes anti-checkpoint administration, the inflammation could proceed to organ toxicity.

Researchers suggest that one of the mechanisms causing autoimmune thyroid disease with pembrolizumab treatment is the formation of autoantibodies (thyroid Peroxisome Antibody (TPOAb) and thyroglobulin antibodies (TgAbs)[28]. Velu *et al.* (2009)[38] assumed that PD-1 plays a vital role in the humoral immune response being presented on the B cell surface. Thereby PD-1 inhibition and involvement of T cells in thyroid tissue injury lead to antigen exposure. The latter activates B-cell to secrete thyroid- antibodies and share in thyroid tissue injury by antibody or complement-dependent pathway[39].

Moreover, Delivanis *et al.* (2017)[30] detected elevated fludeoxyglucose (FDG) uptake of the thyroid gland with pembrolizumab treatment, which is a marker of autoimmune thyroid disease.

In this study, different ultrastructural changes were evident upon administration of pembrolizumab, such as nuclear electron-dense chromatin, destroyed mitochondria and distended RER. These changes point to the direct toxic effect of pembrolizumab on thyroid tissue.

Moreover, cytoplasmic vacuolations demonstrated in our light and electron microscope results refer to apoptosis. Studies indicated that binding of PD-1 to PD-L1 prevents the apoptosis process in cells expressing these receptors by inhibiting T cell activation in these tissues. So, disturbed apoptosis prevention occurs by inhibiting such binding[40]. Hori *et al.* (2020)[41] mentioned that the activated T cells express Fas, which when binds to Fas ligand in the tissue, explores caspase cascade resulting in apoptosis. This was confirmed immunohistochemically by increasing caspase expression in liver cells treated with pembrolizumab[42].

A correlation between tissue destruction and oxidative stress has been proven by Türkmen *et al.* (2023)[42], who found a drop in antioxidants as GSH levels and decreased SOD and CAT upon pembrolizumab administration.

Congestion and leakage of blood from blood vessels in this study come hand in hand with Mekki *et al.* (2018)[43], who discovered hypervascularity in thyroid ultrasound imaging. Angell *et al.* (2018) [33] and Kobayashi *et al.* (2018)[44] confirmed that this hypervascularization shares in the hypoechoic appearance and hypertrophy of the thyroid gland and is also associated with increased thyroid antibodies after treatment with immune checkpoint inhibitors.

Vascular leak occurs due to cytoskeletal endothelial cell distortion from the migration of leukocytes through their gaps to the tissues allowing passage of blood to extravascular tissue[45].

In this study, we found Many follicles were lined by numerous layers of follicular cells, which is defined by Jabkowski *et al.* (2021)[11] as pseudogranulomas formed by the destroyed follicles with the persistence of follicles residual colloid.

Regarding thyroid hormone values, pembrolizumab-treated rats displayed a statistically significant drop in serum total T3 and T4 levels related to the control group. These findings came in agreement with Schmidt *et al.* (2022)[28], who reported pembrolizumab-induced reduction in thyroid hormones, which required replacement with levothyroxine in these cases.

The present study tested for the first time the ability of mesenchymal stem cells conditioned media (MSCs-CM) to improve the structural appearance of thyroid sections and thyroid hormone profile. Most of the histological sections were better than those seen in the pembrolizumab-treated group. In addition, a significant increase in free T4 level was evident compared to the pembrolizumab-treated group.

Researchers recorded that secretomes, microvesicles and exosomes secreted in stem cells conditioned media have antiapoptotic, tissue repair and pro-mitotic effects on cells[46]. Sanchooli *et al.* (2017)[47] found that stem cells conditioned media administration was coupled with amelioration of extracellular matrix (ECM) due to its TGF-β content.

MSCs-CM has captured considerable attention due to many growth factors aiding in restoring cell survival as insulin-like growth factor-1(IGF-1), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFG[48].

Pawitan *et al*. (2014)[49] reported cellular proliferation in conditioned media group due to platelet-derived growth factor (PDGF) secreted.

We found amelioration of cellular organelles structure in the MSCs-CM treated group in the form of nearly normal mitochondria and RER and minimal cytoplasmic vacuolations. MSCs-CM protects the cells from oxidative stress by altering the cell membrane's hydrophobic features, making them no more permeable to hydrogen peroxide, preventing cell death[50].

CONCLUSION

It was concluded that pembrolizumab, the last line of defense for patients with advanced cancer, had immune-related deleterious effects on the thyroid gland that were visible histologically, biochemically and immunohistochemically. Therefore, it should be taken with great caution and regular thyroid monitoring.

MSCs-CM had been promoted as a novel therapeutic agent free of cells for the treatment of many diseases. MSCs-CM use prevents the possible dangers of immunogenicity, tumorigenicity and infection for recipients, among other potential safety issues. From our study, we concluded that MSCs-CM play a therapeutic role to improve PEMB thyroid ultrastructure histological alterations.

RECOMMENDATION

It is recommended to do routine checkup for thyroid hormones, blood pressure and blood glucose levels from the beginning until the end of pembrolizumab treatment. Also, another studies still needed to confirm the therapeutic effect of MSCs-CM.

CONFLICT OF INTEREST

There is no potential conflict of interest among the authors.

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الملخص العربى

الإمكانات العلاجية للوسائط المكيفة بالخلايا الجذعية على التغيرات النسيجية لمثبط نقاط التفتيش المناعية بيمبروليزوماب على الغدة الدرقية للجرذان

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مقدمة البحث: مثبط نقاط التفتيش المناعية البيمبروليزوماب هو جسم مضاد وحيد النسيلة يحفز استجابة مناعية قوية مضادة للأورام. ارتبط قصور الغدة الدرقية وقصور الغدة الكظرية بإضافته إلى بروتوكول العلاج. الوسائط المكيفة هي وسائط استزراع للخلايا الجذعية تشتمل على البروتينات القابلة للذوبان والدهون والأحماض النووية والحويصلات خارج الخلية.

الهدف من البحث: يهدف هذا البحث إلى تحديد ما إذا كانت الوسائط المكيفة بالخلايا الجذعية يمكنها تحسين الاضطرابات النسيجية للغدة الدرقية الناجمة عن البيمبروليزوماب.

المواد والطرق: تم دمج 45 جرذاً ذكراً بالغاً في هذا العمل وتم تصنيفهم إلى: المجموعة الضابطة، المجموعة المعالجة بالبيمبروليزوماب: تم إعطاء البيمبروليزوماب داخل الصفاق بجرعة 3 ملجم / كجم ثلاث مرات في الأسبوع لمدة أربعة أسابيع، مجموعة الوسائط المكيفة عولجت كالمجموعة الثانية ثم تلقت 200 ميكرولتر من الوسائط المكيفة بالخلايا الجذعية عبر الوريد الذيل مرتين في الأسبوع لمدة ثلاثة أسابيع. قمنا بقياس هرمونات الغدة الدرقية وعلامات الأكسدة. تم فحص الغدة الدرقية بالمجهر الضوئي والإلكتروني. تم استخدام الإحصائيات لتحليل جميع البيانات.

النتائج: أظهرت مجموعة البيمبروليزوماب تقشر الخلايا الجريبية في التجويف، والفجوات السيتوبلازمية والمساحات الخلالية الواسعة بين الجريبات. تظهر بعض البصيلات ملتوية، والبعض الآخر يبدو متوسعًا، وبعض البصيلات تفتقر إلى المادة الغروية. تُظهر مجموعة الوسائط المكيفة بالخلايا الجذعية تحسنًا في المظهر الهيكلي لأقسام الغدة الدرقية. غالبية البصيلات مليئة بالغروانية. تصطف الخلايا المكعبة التي تحتوي على نوى مركزية كبيرة مستديرة على غالبية بصيلات الغدة الدرقية. لا تزال بعض الجريبات تحتوي على السيتوبلازم المفرغ.

الاستنتاج: وجد أن الوسائط المكيفة بالخلايا الجذعية لعبت دورًا علاجيًا لتحسين التغيرات النسيجية للغدة الدرقية الناجمة عن البيمبروليزوماب.