OPEN ACCESS Freely available online

Journal of Hepatology and Gastrointestinal Disorders

Research Article

Cell Wall Deficient Mycobacteria (CWDM) in Blood Myelocytes and Brain Tissue in Late Onset Alzheimer's Disease (AD) Implications for New Approaches in Drug Development

William Chamberlin[1*], Andrew Tie[2], Christopher Fowler[3], Lawrence Ward[4], John Aitken[5]

[1]Department of Mycobacteria, Protibea Therapeutics Inc, Texas, USA; [2]Department of Pathology, Wellington Hospital, Wellington, New Zealand; [3]Department of Neurology, The University of Melbourne, Melbourne, Australia; [4]Department of Medicine, The University of Melbourne, Melbourne, Australia; [5]Department of Medical Microbiology, Otakaro Pathways Ltd, Christchurch, New Zealand

ABSTRACT

Introduction: The presence of cell wall deficient Mycobacteria (L-Form) infections in macrophages and Alzheimer brain tissue is not currently recognized.

Methods: Elderly subjects were selected from the Australian Imaging, Biomarker and Lifestyle study of ageing and grouped as MCI, AD and cognitively normal. Blood buffy coats were cultured and stained using new methods. Histologic slides from archived AD brains were stained for the presence of Mycobacteria L-forms.

Results:

MCI=36/36 blood cultures positive

AD=14/14 positive

Elderly controls=52/52 positive

AD Brain Tissue=10/10 samples positive for CWDM

Conclusions: Elderly people have Mycobacteria L-forms in blood macrophages. Patients with Alzheimer's disease have what appears to be the microbe in their brains surrounded by biofilm. Members of the genus *Mycobacterium* join the growing list of microbes associated with AD. Age related immune senescence may enable opportunistic microbes to trigger neuro-inflammation and become pathogenic.

Keywords: Alzheimer's disease; Blood cultures; Drug development; *Mycobacterium*; Crohn's disease

INTRODUCTION

Current concepts do not recognize the presence of CWDM in blood, brain or other tissues. This assumption is being questioned. For example, the Leiden University group report that Mycobacteria form viable cell wall-deficient cells that are undetectable by conventional diagnostics. They conclude that "given that these wall-deficient Mycobacteria are undetectable using conventional diagnostic methods, such cells have likely been overlooked in clinical settings" [1].

The Ziehl-Neelsen staining technique is used to demonstrate Mycobacteria. Ehrlich's modification to the decolouriser will stain all Mycobacteria including Non-Tuberculosis Mycobacteria (NTM) and CWDM [2]. Acid-alcohol decolourisers are suboptimal for this although they are optimised for M. *tuberculosis bacilli*. However, adjustments to concentrations of basic fuchsin in the carbol fuchsin primary stain and changes in the decolouriser allow detection of CWDM in tissue and fluid media [3]. Previously, CWDM in circulating blood myelocytes and in diseased intestinal tissue from Crohn's disease patients were grown and visualized [3]. The role of CWDM in Crohn's disease is linked to immune deficiency genes.

Knowing that AD is a chronic neuro-inflammatory disease that occurs in the elderly as immunity declines, we were curious to determine if a previously unappreciated infection with CWDM could also be present in Alzheimer's patients.

Correspondence to: William Chamberlin, Department of Mycobacteria, Protibea Therapeutics Inc, Texas, USA, E-mail: wchambrlin@aol.com

Received: 08-Mar-2024, Manuscript No. JHGD-24-30074; **Editor assigned:** 12-Mar-2024, PreQC No. JHGD-24-30074 (PQ); **Reviewed:** 27-Mar-2024, QC No. JHGD-24-30074; **Revised:** 04-Apr-2024, Manuscript No. JHGD-24-30074 (R); **Published:**11-Apr-2024. DOI:10.35248/2475-3181.24.10.283

Citation: Chamberlin W, Tie A, Fowler C, Ward L, Aitken J (2024) Cell Wall Deficient Mycobacteria (CWDM) in Blood Myelocytes and Brain Tissue in Late Onset Alzheimer's Disease (AD) Implications for New Approaches in Drug Development. J Hepatol Gastroint Dis.10:283.

MATERIALS AND METHODS

Subject selection

A total of 102 subjects were selected from the Australian Imaging, Biomarker and Lifestyle study for ageing (AIBL), Florey Neurological Institute at University of Melbourne. Of these, 36 were diagnosed with Mild Cognitive Impairment (MCI) and 14 diagnosed with late-stage AD. Fifty-two subjects 65 years or older who had not been diagnosed with either MCI or AD served as controls.

Culture and staining methods

Blood was collected *via* venipuncture into a tube containing sodium citrate as an anticoagulant, sent to Otakaro Pathways in New Zealand and retained at ambient temperature until separation of plasma. The sample was placed into a centrifuge and separated at 3000 RPM for 10 minutes. The plasma collected from the sample was deposited into a sterile collection tube and frozen at minus 2°C. The buffy coat was harvested from the remaining sample and deposited into a MGIT tube supplemented with OADC, Mycobactin J, sucrose 1% and Panta. In addition to the use of the MGIT tube (BD Bactec MGIT Mycobacterial growth indicator tube), considered to be the gold standard for detection of MTBC, we used several in-house media that were optimized for the detection of CWDM. Growth of CWDM is extremely slow and cultures are examined over the longer term in different media to detect growth and changes in morphology. The inoculated MGIT tube was incubated aerobically at 37°C without agitation for at least 30 days and examined at 8 and 30 days. After incubation the buffy coat was harvested and inoculated onto a microscope slide. The slide was then air dried, heat fixed, stained and examined under x1000 oil immersion. One hundred (100) cells were counted.

Alzheimer's brain histology

Slides from archived paraffin blocks containing tissue from Alzheimer's brains were obtained from Duke University and Mayo Clinic. Ten samples were prepared and sent to Dr. Andrew Tie in Wellington, New Zealand for inspection using a variation of the Ziehl-Neelsen stain.

RESULTS

Blood cultures

Examination of blood cultures using a commercial Mycobacterial culture system and modified ZN stain show the presence of small coccoid ZN positive organisms occurring intracellularly in macrophages and extracellularly surrounded by biofilm in all 102 subjects. In addition, CWDM resembling the CWDM seen in brain tissues were isolated from some of the blood cultures (Figure 1).

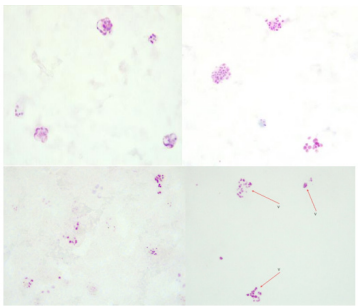

Figure 1: Blood culture: Alzheimer's disease-CWD forms of mycobacteria infect blood myelocytes and can be grown in culture and stained with a modified Ziehl-Neelsen stain. Colonies take on different shapes and sizes.

The specimens were sub-cultured using a proprietary media. After extended incubation they produced micro-colonies. PCR identifies them as *Mycobacterium* species.

Alzheimer's disease brains: Similar appearing findings resembling CWDM are seen in brain tissue under x1000 oil immersion in all ten (10) specimens examined (Figure 2 and 3). PCR identification of these findings was not performed and so they remain just 'highly suspicious findings'. The working diagnosis is that they are CWDM, but the genus or species is not confirmed. Examination of control brains was not performed. Post-mortem contamination cannot be excluded but is unlikely.

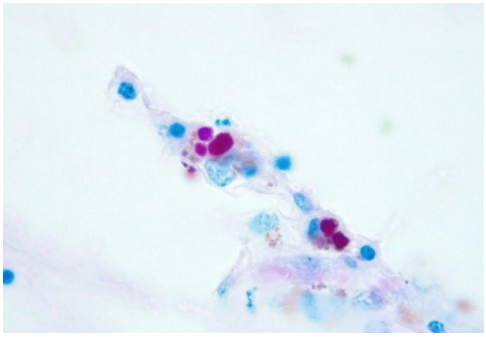

Figure 2: Hippocampus-this area of the brain is associated with AD manifestations.

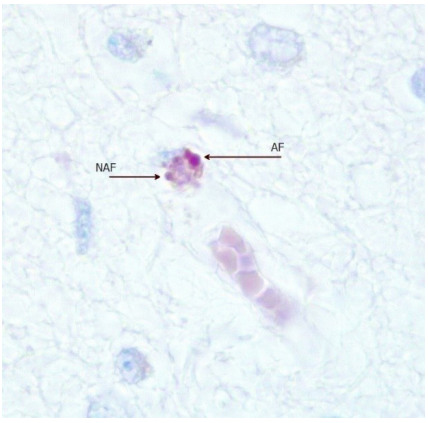

Figure 3: Occipital lobe-Non-Acid fast and Acid-fast forms in biofilm.

Blood cultures of elderly controls *vs.* Mild cognitive impairment *vs.* Alzheimer's: Under microscopic examination, slides from 52 elderly healthy controls, 36 with mild cognitive impairment and 14 with advanced dementia appeared similar in number and morphology. No reliable distinctions could be made between the groups.

Conclusions about the etiologic significance of these findings cannot be made. However, these findings may be important in that they reverse current thoughts that Mycobacteria L-forms do not infect innate immune cells.

Healthy controls: In a previous series of 40 healthy young adults, 39 were also positive. They were all small and infrequent and judged to be the dormant forms. These finding raise the possibility of Mycobacteria being part of an internal blood microbiome (Figure 4).

Brain tissue (cadaver): Findings consistent with CWDM in biofilm were seen in AD brain tissue in 10/10 specimens examined. (DNA confirmation was not obtained from brain specimens nor were healthy brains examined) (Figure 4).

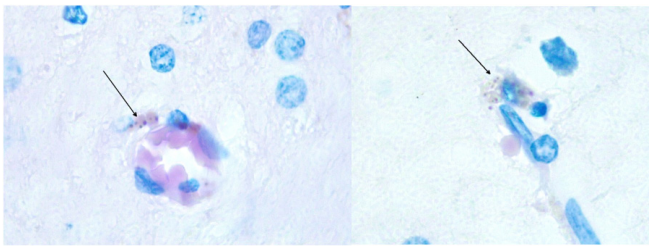

Figure 4: Findings surrounded by biofilm.

Crohn's disease: CWD Mycobacteria in 18/18 cd patients *vs.* 0/15 controls from resected small intestine stored in paraffin (Figure 5).

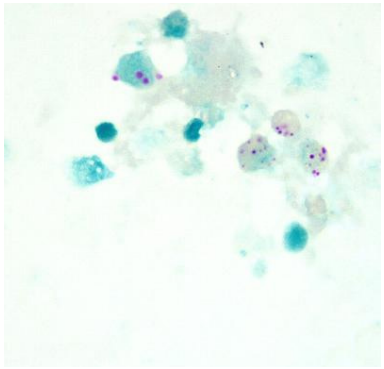

Figure 5: CWD-Mycobacteria are also cultured from blood WBC in other diseases. Blood culture from a patient with Parkinson's disease..

DISCUSSION

Research in context

Newly described culture and staining methods identify CWDM in blood innate immune cells (Figure 6,7 and 8). CWDM were seen in Crohn's disease ileum but not in healthy controls. Similar findings in neuro-inflammatory dementia patients were entertained. Electronic literature screens for 'cell wall deficient Mycobacteria, L-forms and Alzheimer's disease were performed.

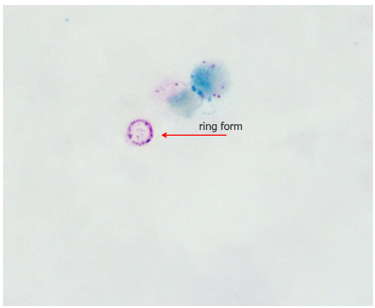

Figure 6: Parkinson's disease blood culture: ZN stain binding to Mycolic Acid in the membrane of CWDM.

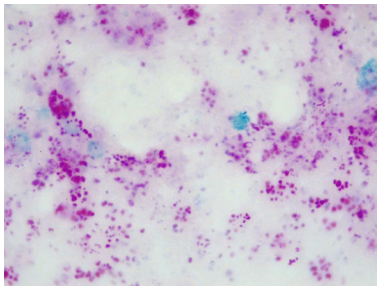

Figure 7: Crohn's disease in clinical flare.

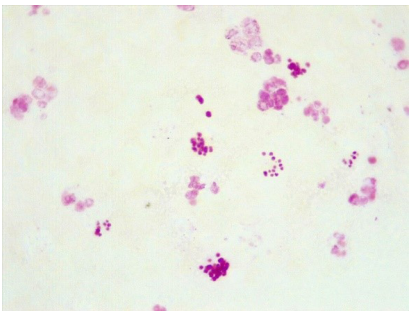

Figure 8: PA variety of shapes and sizes of L-forms from Crohn's disease blood culture.

Current status: The literature discusses cell wall deficient Mycobacteria but its role in disease is largely unappreciated. Current concepts do not recognize the presence of CWDM (L-Forms) in blood, brain or other tissues (Figure 9). Previously undetected CWDM infections could cause idiopathic chronic inflammatory syndromes.

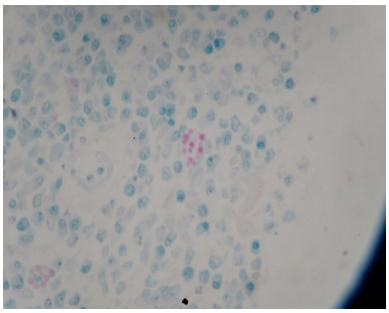

Figure 9: L-forms seen either intracellularly in blood myelocytes or extracellularly in tissue surrounded by biofilm.

Added value: Mycobacteria join the growing list of microbes associated with Alzheimer's disease. Identifying CWDM in circulating innate immune cells is a new finding (Figure 10).

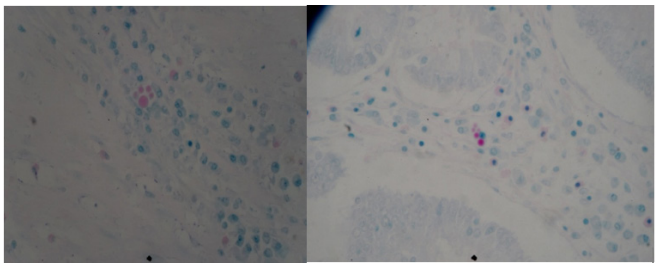

Figure 10: Mycobacteria L-forms in Crohn's disease ileum.

Implications: Treatment strategies should strive to mitigate immunosenescence and inflammaging to restore effective immunity and control infections. Research must determine the role of CWDM as a component of an internal microbiome.

This exploratory study was performed to see if CWDM are present in blood myelocytes in individuals with AD and, if so, are CWDM present in brain tissue? The answer is yes and yes, CWDM infect circulating WBCs and appear to be present surrounded by biofilm in AD brains. Clearly, Mycobacteria infection in the blood is a common, reproducible finding. Small, dormant appearing forms are frequently seen in healthy young individuals.

In brain tissue sections, Non-Acid Fast (NAF) coccoid organisms and Acid-Fast Organisms (AFO) were seen in all 10 patients. Of particular interest were acid-fast organisms associated with

biofilm. Non acid-fast organisms have previously been reported in other tissues which relate to the loss of acid fastness seen on microaerophilic cultures and cavities.

Ziehl-Neelsen stain and cwd forms

The Ziehl-Neelsen stain attaches to mycolic acids in the cell wall, but the absence of a cell wall does not automatically rule out the use of the ZN stain because mycolic acid specifically binds with carbol-fuchsin and the staining reaction does not require a cell wall. Mycolic acid is manufactured in the cytoplasm and is transported across the inner membrane.

Questions arise

Are these bacteria opportunistic pathogens that are present in low numbers during health but cause disease when immunity declines with ageing?

Do they infect tissue-specific macrophages and cause disease in other organs in immune susceptible hosts?

Do they contribute to age-related immune dysregulation and chronic inflammation?

Are Mycobacteria part of a blood microbiome? A brain microbiome? A brain pathobiome?

Our findings show that CWDM infect blood innate immune cells and reveal what appears to be Mycobacterial L-forms in AD brains. The role of the microbes seen in these pictures must now be explained. Their role may be causal, secondary or unrelated.

The *Mycobacterium* genus numbers over 190 species. The majority of the members are distributed across the environment in water, soil and animals. In most instances, contact with Mycobacteria by humans carries no risk and the few that can cause disease are opportunistic pathogens, able to cause disease only in immunocompromised humans and animals. The exceptions are obligate human pathogens such as *Mycobacterium Tuberculosis* Complex (MTBC) and *Mycobacterium* leprae. Despite advances in medicine, *M. tuberculosis* remains one of the leading causes of infectious disease mortality across the globe.

MTB has evolved to cause human disease in contrast to most other members of the *Mycobacterium* genus but other Mycobacteria species may evolve to make the jump from animals to humans.

Humans are in daily contact with non-pathogenic, non-tuberculous Mycobacteria. Continual human exposure to Mycobacteria without initiation of disease has been of longstanding interest to researchers. Mycobacteria share some common antigens across the genus, and it may be that that an environmental vaccination process in humans occurs through ongoing exposure to antigens from commensal Mycobacteria in food and water.

Alzheimer's Disease is associated with other microbial infections. Evidence exists for the presence of viruses, bacteria, protozoa, and fungi in AD brains. Researchers hypothesize the existence of a pathogenic brain microbiome occurring in elderly dementia patients-and perhaps in many elderly patients who remain sub-clinically infected. Other microbes include Herpes, *Mycoplasma* species, *Chlamydiae*, *Treponemes*, *Porphyromonas gingivalis*, SARS-COVID-2, Varicalla zoster virus, *Helicobacter pylori* and undoubtedly others. One should note that microbes as causal agents of AD have long been recognized if one considers neuro-syphilis. *Mycobacterium tuberculosis* is a well-recognized risk factor for AD, providing further incriminating evidence for a role for the *Mycobacterium* genus in neurodegenerative disease. This paper now spotlights the genus *Mycobacterium*.

Our thoughts turn to several roles that CWDM may be playing in Alzheimer's disease.

- They may provide chronic antigenic stimulation that trigger neuro-inflammation.
- They may interfere with immune signaling cascades causing immune dysregulation.
- CWDM in higher numbers can serve as indicators of immune senescence. As biomarkers of immune senescence, they support the need for therapies that enhance immunity.

We are led to a model for the etiopathogenesis of AD that closely follows that proposed by Richard Lathe [4]. This model also applies to other chronic inflammatory diseases of ageing. Ageing is genetically programmed and driven by neuro-circuitry related biological clocks in the primitive brain. Circulating factors (by definition hormones) orchestrate global responses that impact on epigenetic mechanisms [5]. Over time, hormone levels fall (steroids, HGH, FGF etc.) and are associated with declining stem cell function in different organs. The immune system is particularly sensitive to the ageing process resulting in declining immunity and chronic low-grade inflammation. Compromised immunity predisposes to infections by microbes. Microbes that are usually contained by robust immunity are able to elicit or accentuate dysregulated inflammation leading to diseases in different organs.

This model essentially describes immunosenescence and inflammaging associated with infection. Our working hypothesis is that chronic neuro-inflammation is the common pathway responsible for synaptic malfunction, neurodegeneration and Alzheimer's disease.

Therapeutic approaches

Microbes play a role in diseases of ageing [6]. However, a more fundamental and upstream problem lies in declining immunity predisposing to infection. We suggest that treatments that strengthen immunity, downregulate inflammation and control infections are desirable [7]. Members of the steroid metabolome are essential hormonal signaling factors that influence the ageing process. DHEA derivatives and/or their downstream metabolites are prime suspects [8]. Serum DHEA levels peak in the 20's and gradually fall as we age. By 65, levels have declined to 10%-20% of their peak. A therapy that stimulates innate immunity, down-regulates chronic inflammation and has anti-microbial activity against viruses, bacteria, protozoa and fungi would be desirable. The synthetic steroid water-based 16 alpha Bromo-Epi-Androsterone (BEA) has demonstrated these qualities in animal and human studies in other diseases [5,9-11].

BEA was judged to be 60 times more powerful than DHEA in studies on the inhibitory action of BEA in preventing lymphocyte transformation caused by Epstein-Barr Virus. Other immune effects are congruent with this observations [12].

CONCLUSION

In summary, ageing is genetically programmed, orchestrated by neurological 'clocks' in the brain and coordinated globally through circulating factors (hormones) influencing epigenomic DNA-methylation and gene programs. Declining hormone signaling underlies immune senescence and infections by opportunistic

microbes. We demonstrate Mycobacteria in blood myelocytes and brains from patients with Alzheimer's disease. Mycobacteria join the list of potential microbes involved in Alzheimer's disease. A promising therapeutic strategy would correct deficient hormonal signaling and thereby enhance immunity, down-regulate inflammation, control infections and mitigate neuro-inflammatory processes.

ACKNOWLEDGEMENTS

- Data used in the preparation of this article were obtained from the Australian Imaging Biomarkers and Lifestyle flagship study of aging (AIBL), funded by the Commonwealth Scientific and Industrial Research Organization (CSIRO), National Health and Medical Research Council (NHMRC), and participating institutions. AIBL researchers are listed at www.aibl.csiro.au. The authors thank all participants who took part in the study, as well as their families.
- Extensive microbiological work performed by Robin Phillips, Jack E. Aitken and David S. Aitken was essential for this project.
- Improved techniques discovered by Khoi Phan enabled successful staining of cell wall deficient Mycobacteria in blood and brain specimens.
- In memory of Professor John Hermon-Taylor: Scientist, clinician and role model. John's passionate pursuit of scientific truth in this field will forever influence all that follow.

CONFLICT OF INTEREST

Chamberlin W: Co-founder of Protibea Therapeutics, a company advancing an Immune Regulating Hormone for the treatment of Alzheimer's disease.

Moriarty J: Co-founder of Protibea Therapeutics, a company advancing an Immune Regulating Hormone for the treatment of Alzheimer's Disease.

Aitken J: Financial interest in Otakaro Pathways which he founded. He developed culture media to grow Non-Tuberculous Mycobacteria and stain Mycobacteria L-forms.

Tie A: Nothing to declare.

Fowler C: Nothing to declare.

Ward L: Nothing to declare.

FUNDING

This project was conceived and funded by James Moriarty and William Chamberlin, principals of Protibea Therapeutics. Funds come from Angel Investors.

AUTHOR CONTRIBUTION

Chamberlin W-WC was involved in conceptualization, original draft, writing and funding the manuscript.

Moriarty J-JM was involved in conceptualization, original draft, writing and funding the manuscript.

Aitken J-JA was involved in conceptualization, original draft, writing the manuscript and performed the labwork involved in the study.

Tie A-As a pathologist, AT oversaw the staining and reviewed and interpreted the brain specimens.

Fowler C-As Research Officer at Florey Neurological Institute, CF was key to the role played by the Australian Imaging, Biomarker and Lifestyle study for ageing.

Ward L-LW was essential for organizing and obtaining data from AIBL.

ETHICS COMMITTEE APPROVAL

Approval from Australian Imaging, Biomarker and Lifestyle study of ageing at University of Melbourne.

REFERENCES

1. Dannenberg N, Carrion Bravo VJ, Weijers T, Spaink HP, Ottenhoff THM, Briegel A, et al. Mycobacteria form viable cell wall-deficient cells that are undetectable by conventional diagnostics. BioRxiv.2022.
2. Aitken JM, Borody TJ, Agrawal G. A revaluation of the use of conventional Ziehl-Neelsen stain for detection of non-tuberculous mycobacteria. N Z J Med Lab Sci. 2019;73(3):85-86.
3. Aitken JM, Phan K, Bodman SE, Sharma S, Watt A, George PM, et al. A *mycobacterium* species for Crohn's disease?. Pathology. 2021;53(7):818-823.
4. Lathe R, St Clair D. Programmed ageing: Decline of stem cell renewal, immunosenescence, and Alzheimer's disease. Biol Rev Camb Philos Soc.2023;98(4):1424-1458.
5. Targeting Immune Dysregulation in Alzheimer's Disease. Protibea Therapeutics Inc. 2022.
6. Lathe R, Schultek NM, Balin BJ, Ehrlich GD, Auber LA, Perry G, et al. Establishment of a consensus protocol to explore the brain pathobiome in patients with mild cognitive impairment and Alzheimer›s disease: Research outline and call for collaboration. Alzheimer's Dement. 2023; 19(11): 5209-5231.
7. US10836788B2. Aqueous suspension compositions, formulations, and water dispersible dry compositions comprising 16alpha-bromo-3beta-hydroxy-5alpha-androstan-17-ketone and hydrates, derivatives, and analogs thereof. Sd Chem Inc. 2022.
8. Khorram O, Lan Vu, Yen SS. Activation of immune function by De-Hydro-Epi-Androsterone (DHEA) in age-advanced men. J Gerontol A Biol Sci Med Sci. 1997;52(1):M1-M7.
9. López-Torres MO, Marquina-Castillo B, Ramos-Espinosa O, Mata-Espinosa D, Barrios-Payan JA, Baay-Guzman G, et al. 16α-Bromoepiandrosterone as a new candidate for experimental diabetes-tuberculosis co-morbidity treatment. Clin Exp Immunol. 2021;205(2):232-245.
10. Nicoletti F, Conrad D, Wang A, Pieters R, Mangano K, Heeckeren AV, et al. 16α-Bromoepiandrosterone (HE2000) limits non-productive inflammation and stimulates immunity in lung. Clin Exp Immunol. 2009;158(3):308-316.
11. Stickney DR, Novelic Z, Garsd A, Destiche D, Frincke J. Safety and activity of the immune modulator HE2000 on the incidence of tuberculosis and other opportunistic infections in AIDS patients. Antimicrob Agents Chemother. 2007;51(7):2639-2641.
12. Henderson E, Schwartz A, Pashko L, Abou-Gharbia M, Swern D. Dehydroepiandrosterone and 16 alpha-bromo-epiandrosterone: Inhibitors of Epstein-Barr virus-induced transformation of human lymphocytes. Carcinogenesis. 1981;2(7):683-686.