Histol Histopathol (2022) 37: 431-439

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE **Open Access**

Histopathological evaluation of insulin-DMSO formula designed for direct nose-to-brain delivery

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Summary. The combination of insulin and DMSO is a patented (Publication No US8987199B2), noninvasive, pharmaceutically strategized preparation for direct nose-to-brain delivery (DN2BD) suggested for the treatment of Alzheimer's disease (AD). Although its main ingredients have been individually researched, no histopathological investigations have been conducted to address this combination effect on the CNS and nasal tissues in animals. The present work was, therefore, designed to investigate the potential histopathological changes induced by this new pharmaceutical combination using a newly developed refractory staining method. The findings presented herein showed no signs of treatment-related lesions or behavioral changes in Sprague Dawley rats following a three-month successive treatment with two strengths of the formula.

Key words: Histopathology, Insulin, DMSO, Alzheimer's disease, Nose-to-brain drug delivery, Fluoresceine Sodium

Introduction

A multitude of pharmaceutical compounds and delivery systems have been designed and investigated with the aim of improving functional recovery after onset of neurodegenerative disorders such as Alzheimer's disease (AD), stroke, Parkinson's disease, multiple sclerosis, spinal cord injuries, and traumatic brain injuries (Yiannopoulou and Papageorgiou, 2013; Md et al., 2018). Additionally, numerous animal and human studies have underscored the fact that therapeutics, including macromolecules like insulin, can reach directly to the brain through the roof of the nose bypassing the blood brain barrier (BBB) and the systemic circulation as they travel along the olfactory, trigeminal pathways and nerve fibers (Hanson and Frey, 2008; Abdel Maksoud and Maher, 2015; Bourganis et al., 2018). This route was, therefore, dubbed "direct nose-to-brain delivery" (DN2BD). Furthermore, previous reports indicated that intranasally delivered insulin decreases the cognitive decline associated with AD and other neurodegenerative diseases (Reger et al., 2008; Dhuria et al., 2010; Alexander and Saraf, 2018). Dimethyl sulfoxide (DMSO), a widely used vehicle in biological studies and pharmaceutical formulations was also used as a drug carrier in AD-targeted formulations such as tau kinase and glycogen synthase kinase-3β inhibitors (Julien et al., 2012).

Moreover, DMSO dissolves both polar and nonpolar compounds and is miscible with a wide range of organic solvents as well as with water and has an inhibitory effect on beta-amyloid peptides (Aβ) by altering its biological activity through its solubilizing potential (Penazzi et al., 2017). Additionally, the efficacy of DMSO in CNS-related disorders including AD was previously suggested based on its ability to enhance cranial blood flow, inhibit cholinesterase activity along with inhibiting β-amyloid deposits (Kumar and Darreh-Shori, 2017).

Although approved therapies for AD and other neurodegenerative disorders exist, they are categorized as symptomatic treatments for mild to moderate cases (Pires et al., 2009). The scarcity of disease modifying drugs that are capable of blocking the progression of AD necessitates further research to provide therapeutic

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DOI: 10.14670/HH-18-421

Abbreviations. AD, Alzheimer's disease; Aβ, Beta-amyloid peptides; BBB, Blood brain barrier; DMSO, Dimethylsulphoxide; DN2BD, Direct nose-to-brain delivery; FS, Fluoresceine Sodium; H&E, Hematoxylin and eosin; insulin-D, Insulin-DMSO; MB, Methylene blue; NRSM, New refractory staining method; NC, Normal control; SD, Sprague Dawley; SC, Staining control; T, Treated.

options with better outcomes. Intriguingly, in the registered patent for the new drug combination, insulin-DMSO (insulin-D), significant improvement in cognition and memory retention after nasal administration in AD model senescence accelerated mice was demonstrated (Abdel Maksoud and Maher, 2015).

Accordingly, the present work was designed to investigate the potential histopathological effects of insulin-D applied via DN2BD, using a new refractory staining method (NRSM) to enhance visualization of insulin-D induced changes, thereby providing insights on the safety profile of such a combination. This study investigated the likely histopathological changes in the CNS and nasal tissues after DN2BD of two strengths of Insulin-D (4 IU and 6 IU) for three consecutive months.

Materials and methods

Preparation of insulin-D and FS-stained formula

The tested formula comprised of insulin and DMSO where the latter was added as a bioactive solvent, drug carrier (transmitter), absorption enhancer, drug stabilizer and therapy potentiating agent (Bourganis et al., 2018). Recombinant human insulin powder (Sanofi Aventis. GmbH) was pre-complexed with zinc chloride solution to produce the active and readily soluble form of a stable hexamer insulin zinc which is soluble in a synergistic mixture of co-solvents. Phenol was added in the required volume (0.015% v/v) as a stabilizing agent and preservative. When added to the insulin-Zn, phenol produces rhombohedral crystals of two Zn^{2+}-insulin and four Zn^{2+}-insulin hexamer molecules organized as three dimers causing the stabilization of these new helical conformations (Derewenda et al., 1989; Rowe et al., 2009). The insulin-D vials were assigned identification letters where preparation (A) contained 4 IU insulin + 0.04% DMSO, and preparation (B) contained 6 IU insulin + 0.06% DMSO (Table 1).

Another preparation was made by mixing the aforementioned formulae with Fluoresceine Sodium (FS) to provide a staining control in order to validate the successful delivery of insulin-D to the CNS using differential staining protocol with methylene blue (MB) as will be described later.

Animals

Sprague Dawley (SD) rats were used for the current study. They were obtained and housed in the animal house facility of Theodor Bilharz Research Institute. SD rats are characterized by a well-defined nasal cavity that eases the administration of intranasal preparations (Sengupta, 2013). Six-week-old healthy SD male (M) and female (F) rats with an average weight of 150-200 gm were used for the current study. Weights were regularly monitored and recorded. The used rats were germ- and pathogen-free with defined nasal flora and were maintained in separate cages, in a ventilated room at a constant temperature of 24°C, humidity of 50%, light dark cycle (12 h light/12 h dark), and provided with standard commercial diet and water ad libitum.

Experimental design

SD rats were randomly allocated to the following groups (n=6; 3 F and 3 M): (i) Negative control group (NC) in which rats received no treatment. (ii) Staining control group (SC) in which rats were given one time treatment with FS-stained insulin-D in order to detect and validate the absorption and distribution of the formula in the CNS and nasal tissue using a light microscope. Rats belonging to this group were sacrificed on the same day. The remaining rats received the two formulae of insulin-D (A and B), prepared as described in the previous section, intranasally and were assigned different groups based on the time of termination/duration of treatment, as follows: (iii) T1: This group received formula A and was sacrificed after 4 weeks; (iv) T2: This group received formula B and was sacrificed after 4 weeks; (v) T3: This group received formula B and was sacrificed after 8 weeks; and (vi) T4: This group received formula B and was sacrificed after 12 weeks. All treated groups received 2 and 3 ul/nostril for formulae A and B, respectively, twice a day three times per week. Also, a group of SD rats receiving only respective amounts of DMSO were included in the study to ensure the safety of the used concentration. By the end of the experiment, rats were sacrificed under anesthesia using ketamine (80 mg/kg) and brain and nasal tissues were collected for subsequent histopathological examination. It is noteworthy that after 4 weeks of treatment, the histopathological changes of formula A- and B-treated groups were compared and since no changes were observed in the former group having the lower strength, treatment with formula A was discontinued. The experimental design is illustrated in Fig. 1. All experiments were carried out according to the National Institutes of Health guide for the care and use of Laboratory animals and were approved by the Ethics

Table 1. Concentrations and doses of insulin and DMSO in the formulae and the respective frequency of administration for insulin-D.

Insulin-D formula	Insulin concentration	Dose per nostril	Total dose received per rat	Frequency
A	insulin (4 IU)	2 µL	Insulin (4 IU)-DMSO (0.04%)	Twice daily, 3 times per week
B	Insulin 6 (IU)	3 µL	Insulin (6 IU)-DMSO (0.06%)	Twice daily, 3 times per week

Committee of Theodor Bilharz Research Institute.

Intranasal drug delivery

The intranasal drug delivery process was done in the following manner: One rat at a time was removed from the cage, restrained using a modified scruff in the non-dominant hand with the neck held parallel to the floor at a 45-degree angle. Using a micropipette, the formula was intranasally delivered. The droplet was placed deep enough into the rat nostril for inhalation. The rat was held in that position for 15 seconds. The dosing process was repeated on the other nostril. Before the rat was returned to its cage, the nest was restored to its original status to reduce possible stress. After each intranasal dosing session, the rat was given a treat as a reward, then put back in its cage until the next dosing session. Gloves used in the dosing process were discarded after one use to avoid triggered aggressiveness due to new scents.

Behavioral changes and overall health status

General health and physical or behavioral changes in animals were closely and regularly monitored. Excitability, vitality, the amount/frequency of urination, defecation, trembling, and biting, were used for general neurological assessment of the animals. The acclimation of rats to handling, prior to proceeding, was confirmed to ensure successful intranasal drug delivery.

Blood glucose estimation

Blood glucose levels of rats were measured before and one hour after intranasal administration to ensure the bypassing of the blood circulation. Blood glucose level was estimated using a standard glucometer by pricking the tails of rats.

Histopathological evaluation using NRSM

The histopathology protocol for this study was designed to microscopically evaluate the drug-induced effects of insulin-D and all its inactive ingredients on the CNS and nasal tissues using the newly developed refractory staining method (NRSM). It is a three-step differential staining protocol which includes the following: (1) Staining the drug with a suitable chemical marker, namely, Fluoresceine Sodium (FS) to detect successful drug delivery as well as intranasal absorption and distribution of the drug in the brain. (2) Additional staining with a second dye, namely, methylene blue (MB) to produce additional differential color contrast to the routine staining method. (3) Routine tissue staining using hematoxylin and eosin (H&E).

FS, a yellowish-brown dye that has an affinity to acidic and basic body tissues and fluids, was added to

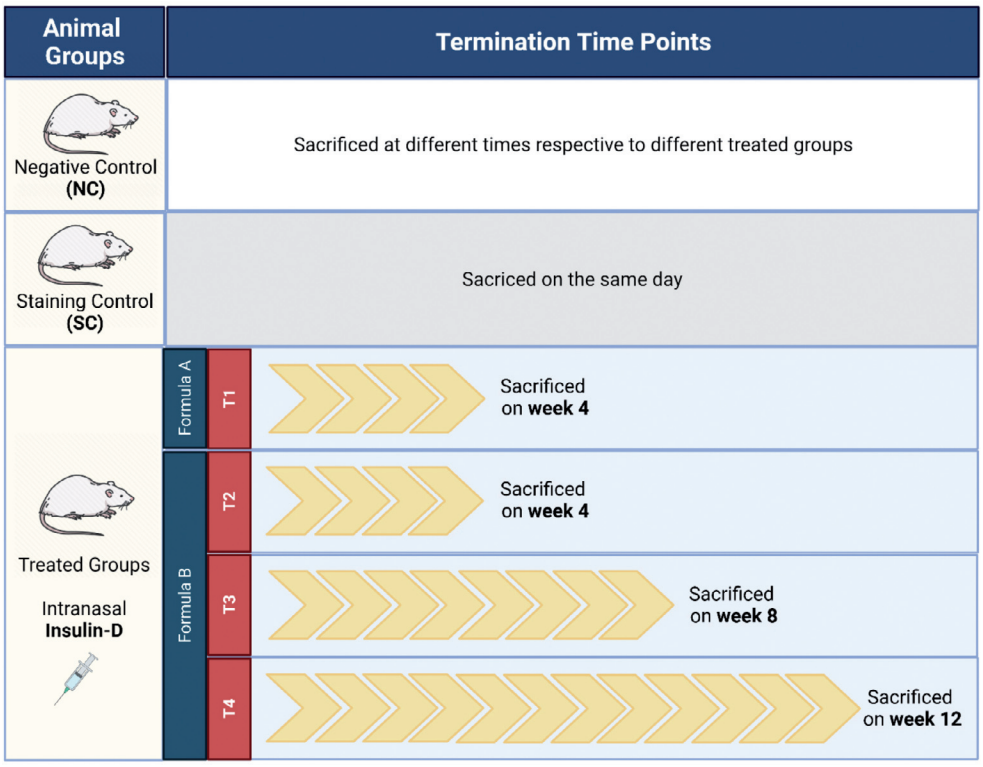

Fig. 1. Experimental design. Illustration of the different groups included in the study along with the different termination time points for each.

the NRSM staining protocol as a chemical marker to detect the absorption and distribution of the formula throughout the CNS and nasal tissues. On the other hand, MB, a basic dye that interacts with cell nuclei staining them blue, was used with FS to create a differential color in the extracellular fluids of the CNS and nasal tissues. The use of FS in this study was derived from the staining procedures used in biochemical research, medical use in urology, ophthalmology, enterology and oncology (Miki et al., 1985; Brancroft and Gamble, 2008; Gurcan et al., 2009; Alturkistani et al., 2015).

Slide preparation

After sacrifice, tissues from the CNS and nasal mucosa were immediately fixed in 10% buffered formaldehyde, then dehydrated with different grades of alcohol (70%, 90%, and 100%) followed by cleaning using xylene. The tissues were embedded in paraffin blocks, then refrigerated for 10-15 minutes before being mounted on a microtome, sectioned at a thickness of 4 µm, placed on clean labeled glass slides, stained with H&E, and then covered (Brancroft and Gamble, 2008; Gurcan et al., 2009; Slaoui and Fiette, 2011).

The control slides and the treated tissue slides were comparatively examined for any lesions or damage to the cerebellum, spinal cord, structural disorganization, edema, pyknotic cells, vacuolization, necrosis of nerve cells (neuronal cells), nasal mucosal oedema, congestion, hemorrhage, ulceration, inflammation, and fibrosis using the computerized image analysis system of AxioVision version 4.8 (Zeiss, Germany).

Statistical analysis

For blood glucose levels, data are presented as means ± S.D. Statistical analysis was performed using Student's t test for unpaired data using GraphPad prism, ver. 8 (CA, USA). Significance was considered at $P<0.05$.

Results

Effect of Insulin-D on blood glucose levels

To ensure that insulin-D preparations had bypassed the systemic circulation, blood glucose levels were measured. As shown in Fig. 2, blood glucose levels of treated groups were not significantly different from normal untreated rats confirming the aforementioned assumption.

Effect of Insulin-D on overall health in animals

No mortalities were reported in the groups treated with insulin-D and none of the rats in any group showed signs of drug-induced neurological disorders. Excitability and vitality were all normal as well as all other signs. Additionally, animals showed normal increases in weights as they grew older during the study.

Effect of insulin-D on histopathology

No signs of drug-induced neurological disorders were observed under the light microscope confirming the safety of preparations. Moreover, both preparations (A and B) were found to cause no drug-induced pathological changes in the CNS or nasal tissues after 12 weeks of DN2BD. Also, none of the rats showed signs of degenerative changes such as congestion of cerebral blood vessels, edema vacuolations, cellular infiltration, nor any signs of minute spinal cord hemorrhages nor dispersed astrocytes. The nose epithelium and olfactory nerves showed no necrosis and no degenerative changes. Additionally, successful D2NBD was validated using FS together with MB for differential staining of insulin-D which was clearly visualized under the microscope, as shown in Fig. 3 and hence endorsing successful delivery

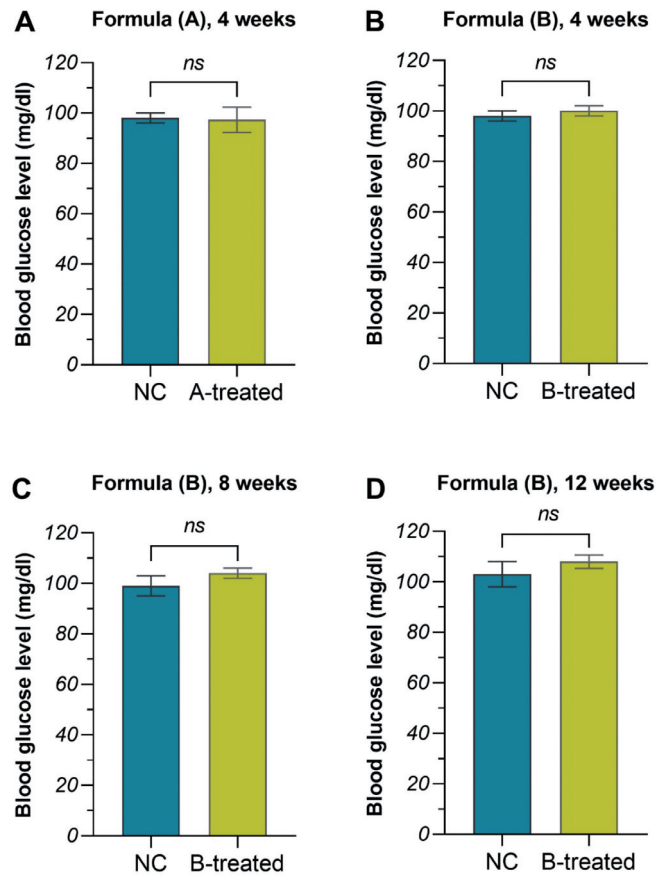

Fig. 2. Effect of insulin-D on blood glucose level. Blood glucose levels of rats receiving Formula A **(A)** after 4 weeks as well as Formula B after 4 **(B)**, 8 **(C)**, and 12 weeks **(D)**. Data are presented as means ± SD. Statistical analysis was performed using Student's t test for unpaired data. Significance was considered at $P<0.05$. ns=nonsignificant.

to the CNS.

Histological sections prepared from brain tissues and stained with H&E belonging to the NC group and insulin-D-treated group (T4) demonstrated no pathologic changes in the CNS after DN2BD (Fig. 4). The sections demonstrated normal histological features of the cerebellum as well as a well-defined molecular layer and the presence of numerous tightly packed small cells in the granular layer and large Purkinje cells (Pyramid cells) in the Purkinje cell layer. Moreover, normal structure of neuronal cells, normal histological structure of the hippocampus and normal structural appearance of the nerve cells in sections taken from the cerebral cortex were evident. Fig. 5 shows comparisons of sections taken from the spinal cord of the NC group and insulin-D-treated group (T4) where normal structure of neuronal cells of the spinal cord was conspicuous.

Additionally, Fig. 6 demonstrates microscopic images from nasal tissues of NC rats showing ciliated respiratory epithelium lining the septum in the anterior nasal cavity illustrating the respiratory epithelium comprising tall columnar goblet cells and basal cells. Submucosal glands are adjacent to the underlying cartilage. Additionally, a section from the nose opening lined by stratified squamous epithelium and submucosal glands were demonstrated. Olfactory epithelium (olfactory cell nuclei), lamina propria which contains blood vessels, lymphatics, and abundant branches of the trigeminal sensory neurons were also shown. The same figure shows the nasal tissues from insulin-D treated rats (T4 group) demonstrating the same features observed in the NC group with no change.

It is worth mentioning that histological sections from other treated groups (T1, T2 and T3) were examined and compared to that of the NC group as well, however, no changes were observed (data not shown). Also, rats belonging to the NC group and receiving DMSO alone did not show any changes when compared to normal rats.

Discussion

Therapeutics, including both small and macromolecules, like insulin, may be targets for rapid and direct delivery to the CNS owing to the unique

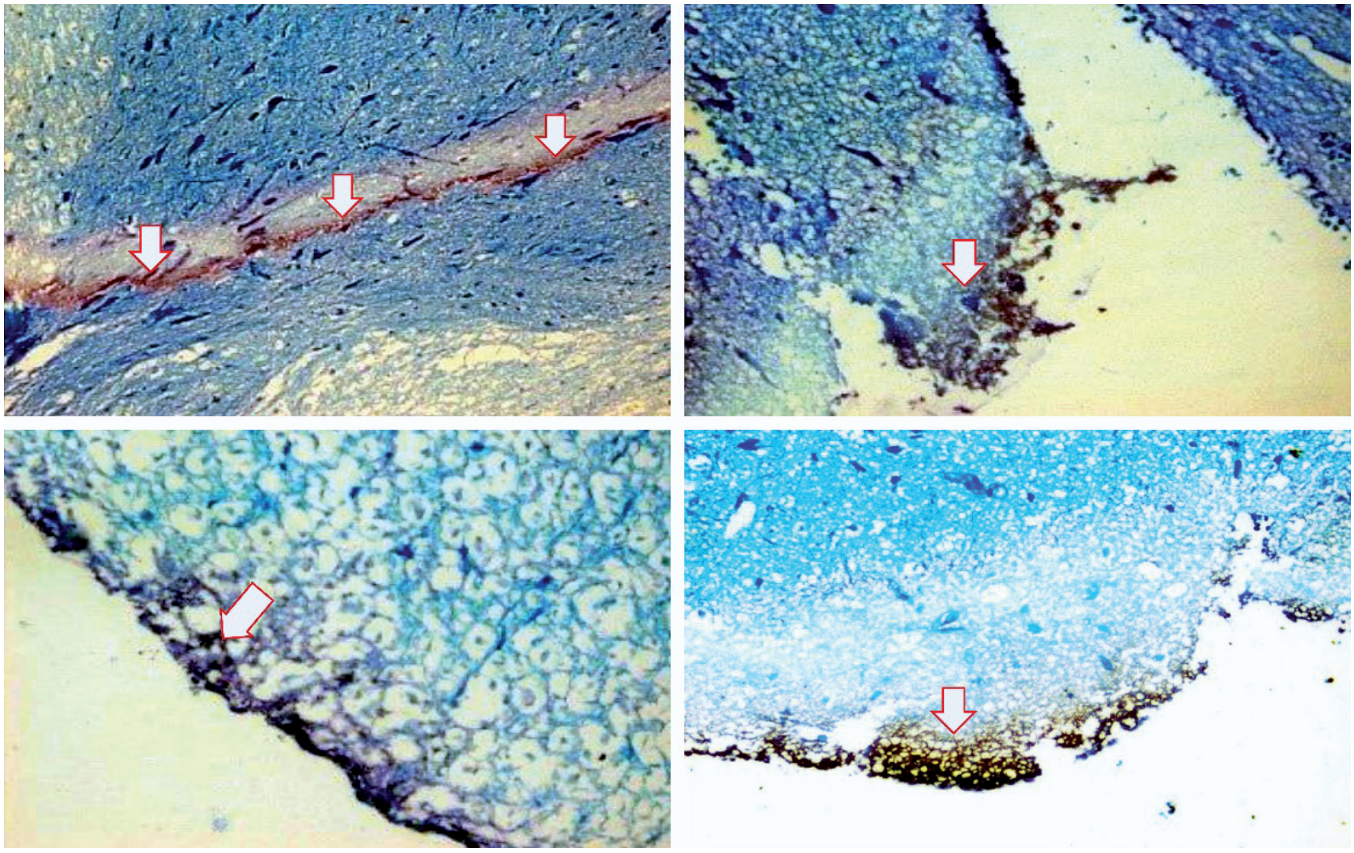

Fig. 3. New refractory staining method (NRSM) applied to brain tissues. Representative photomicrographs from the brains of the negative control group (NC) showing the brownish infiltrate indicating that Fluoresceine Sodium (FS)-stained insulin-D reached the brain tissues after 1 hour of intranasal application. x 200.

connections that the olfactory and trigeminal nerve fibers possess. Moreover, by virtue of the fast onset and bypassing the systemic circulation it offers, DN2BD is considered a sought-after alternative to the conventional routes targeting brain disorders, in general, and neurodegenerative diseases, in particular.

Multiple factors contribute to the success of DN2BD, however, among the most important ones are drug stability, solubility, absorbability, and penetrability. As previously reported, intranasal administration of insulin-DMSO delivers it directly to the brain and spinal cord (Publication No US8987199B2). This method does not target either the lungs (like inhalational products for bronchial asthma), the nose (like antiallergics) or the

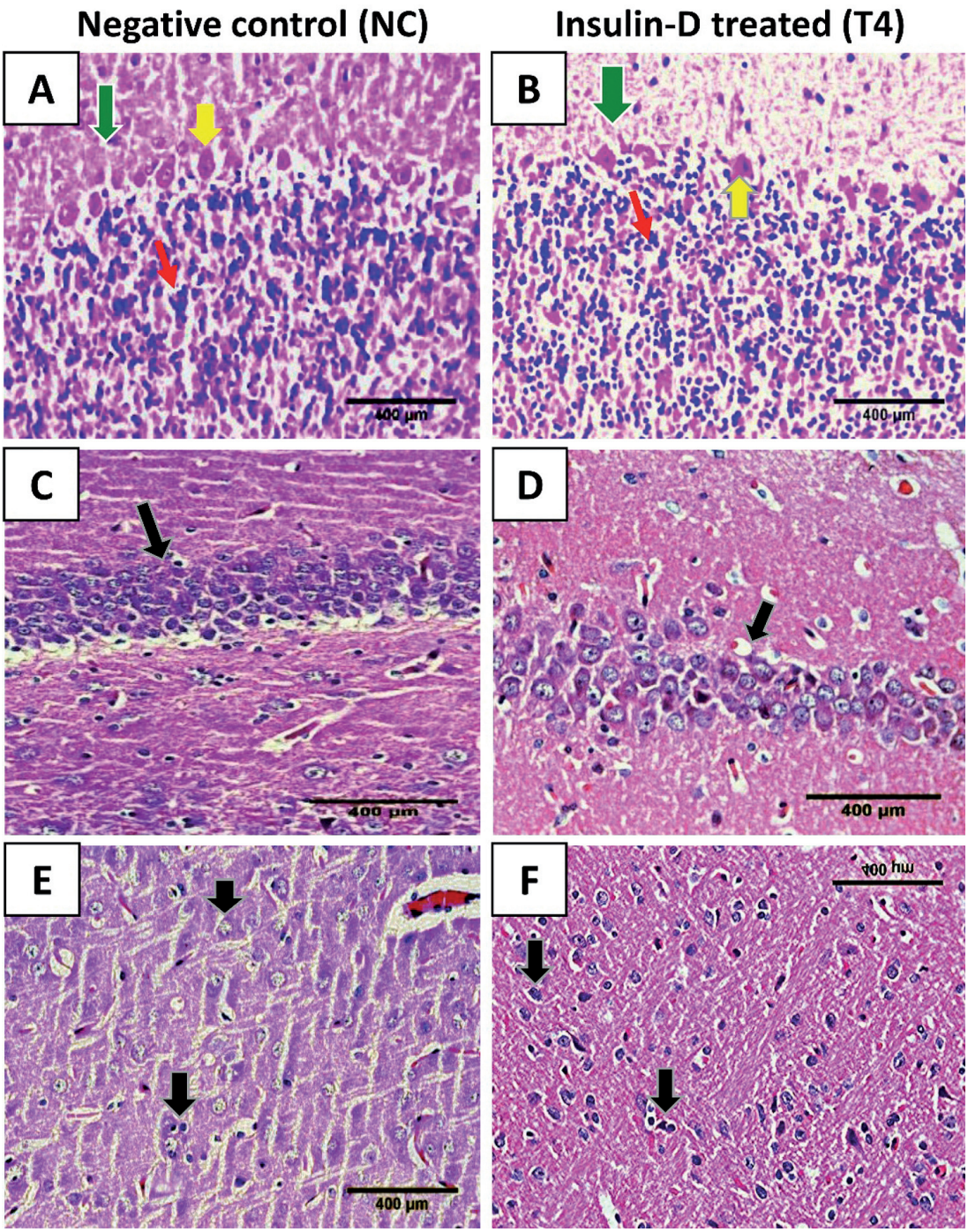

Fig. 4. Effect of insulin-D on cerebellum, hippocampus, and cerebral cortex tissues. H&E-stained histological sections from negative control group (NC) and insulin-D treated group (T4) receiving formula B for 12 weeks, illustrating no pathologic changes in the CNS tissues. **A-B.** Cerebellum with normal histological features, illustrating a well-defined molecular layer and presence of numerous tightly packed small cells in the granular layer (red arrow) and large Purkinje cells (Pyramid cells) in the Purkinje cell layer (yellow arrow), with normal structure of neuronal cells (green arrow). **C-D.** Hippocampus showing normal histological structure (black arrow). **E-F.** Cerebral cortex showing normal structural appearance of the nerve cells (black arrow).

blood stream in the nasal mucosa. Alternatively, intranasal administration of the present pharmaceutical composition reaches the brain and spinal cord along the olfactory and trigeminal fibers as it travels through the roof of the nose.

Interestingly, intranasal drug delivery offers an improved method for the treatment of AD and other neurodegenerative disorders since some routes of drug administration are impractical in such diseases when used in humans, based on convenience, customer compliance, safety, and cost-effectiveness. DN2BD could be considered a local application of pharmaceutically suitable drugs to an organ of a much higher level of sensitivity, and a way of drug delivery bypassing the BBB and systemic circulation to treat brain diseases with far less or negligible peripheral side effects. Thus, it is an innovative, non-invasive, and practical method circumventing the possible risks associated with the oral, rectal, parenteral, or pulmonary administration and the side effects that may accompany the systemic exposure to these pharmaceuticals (Jogani et al., 2008; Pardeshi and Belgamwar, 2013; Kumar et al., 2017).

Insulin-D is a pharmaceutically formulated combination, based on the pathological background of AD characterized by changes involving Aβ and neurofibrillary tau tangles that end up by with the loss of neurons and their connections. Insulin, as the main active ingredient and as an established drug, has no toxic effects on the brain while improving cognitive functions in AD patients (Avgerinos et al., 2018). Meanwhile, DMSO was included for the aim of solubilizing Aβ (Shen and Murphy, 1995; Chertkow et al., 2008; Hanson and Frey, 2008; Abdel Maksoud and Maher, 2015; Penazzi et al., 2017). This histopathological study, therefore, focused on its effects on the CNS and all relevant sites of absorption.

DMSO was approved for use in pharmaceutical formulations in the U.S. and other countries. It was also placed in the safest category, namely, class 3 solvents, with low toxic potential. Class 3 includes no solvent known as a human health hazard at levels normally accepted in pharmaceuticals. Solvents in Class 3 may be regarded as less toxic and of lower risk to human health. It is considered that amounts of these solvents of 50 mg/day or less, corresponding to 5,000 ppm or 0.5%, would be acceptable without justification. Higher amounts may also be acceptable provided they are realistic in relation to manufacturing capability and good manufacturing practice (GMP). Despite being toxic in high concentrations, DMSO has a well-established safety profile in low concentrations and was successfully included in formulations at higher concentrations than the one used in the current study without demonstrating any signs of toxicity (Abhyankar et al., 2017).

The aim of the current study was to assess the drug-induced histopathology of all ingredients constituting the formula on the brain and nasal tissues. The evidence reported herein as well as the results documented in the patent, showed that the formula had successfully reached all CNS regions, based on the findings of the FS-stained group (SC group) without causing any damage to the brain architecture. Also, our findings showed that no drug-induced morphological changes were microscopically observed after intranasal administration of insulin-D using the formulae for three successive months. Thus, we can conclude that even the higher strength of insulin-D (with higher insulin and DMSO concentrations) did not elicit histological changes that

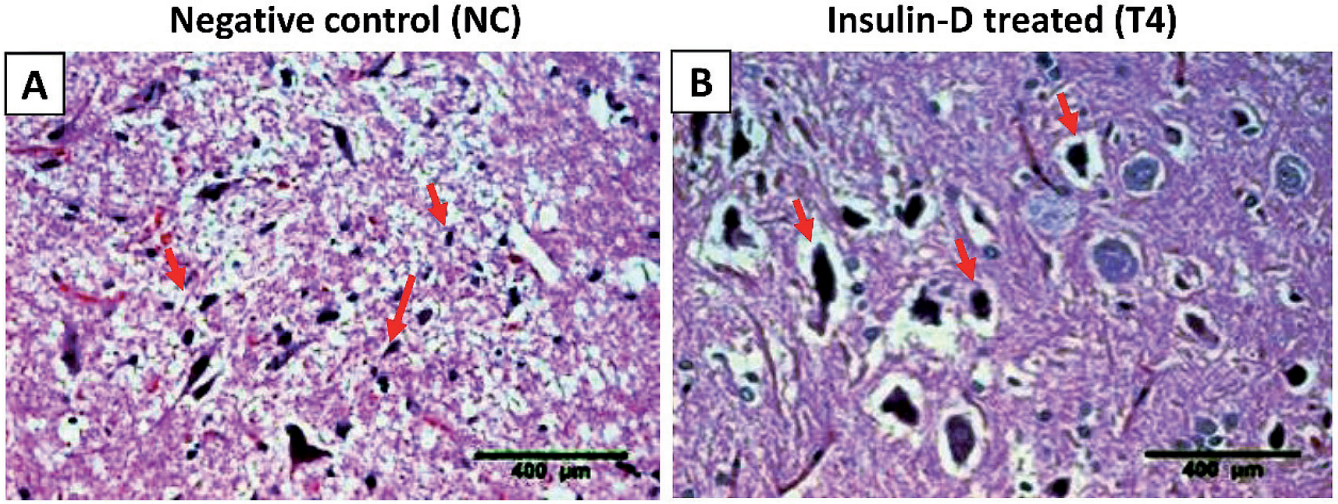

Fig. 5. Effect of insulin-D on the structure of the neuronal cells of the spinal cord. H&E-stained histological sections from negative control group (NC) **(A)** and insulin-D treated group (T4) **(B)** receiving formula B for 12 weeks, illustrating normal structure of neuronal cells of spinal cord.

might imply toxicity when compared to the normal untreated group. Meanwhile, the current study may suggest using the NRSM to the histopathological evaluation protocols as well as FS concurrently with image enhancer techniques as a marker in histopathological and pharmacokinetic studies of drugs administered via DN2BD. Finally, whether DN2BD can be used in the treatment of other brain diseases, particularly brain tumors, needs to be addressed in future studies.

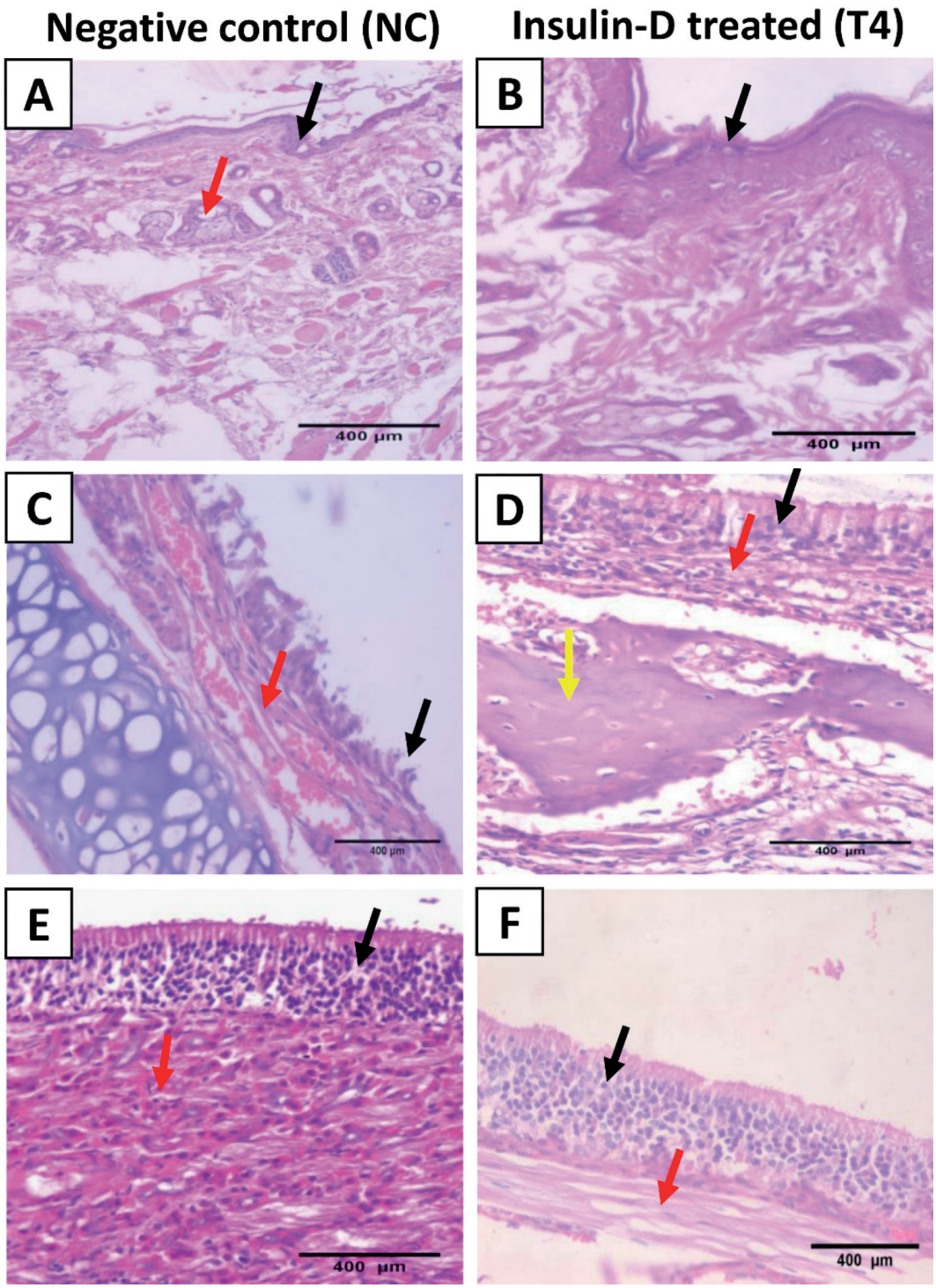

Fig. 6. Effect of insulin-D on the nose histology. H&E-stained histological sections from negative control group (NC) and insulin-D treated group (T4) receiving formula B for 12 weeks, demonstrating nose opening lined **(A, B)** by stratified squamous epithelium (black arrow) and submucosal glands (red arrow). **C, D.** Nose section showing ciliated respiratory epithelium lining the septum in the anterior nasal cavity of rats. The respiratory epithelium consists of tall columnar goblet cells and basal cells (black arrow). Submucosal glands (red arrow) are adjacent to the underlying cartilage (yellow arrow). **E, F.** Olfactory nasal mucosa showing olfactory epithelium (olfactory cell nuclei) (black arrow), lamina propria (red arrow) which contains blood vessels, lymphatics and abundant branches of the trigeminal sensory neurons.

Acknowledgements. Our thanks go to Dr. Yasser R. Abdel-Fattah, the Deputy Minister of Higher Education for Scientific Research Affairs in the Egyptian Government, Dr. Wafaa A. Kandeel, President of TBRI research center, and Yaser A. Abdel Maksoud, Neurosurgeon for his contribution of resources as the CEO of Nerve Access Inc.
Conflict of interest. The authors declare no competing interest.

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Accepted January 18, 2022