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Histopathological study of different organs of charles foster strain rat under the exposure of *Pueraria tuberosa*.

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Abstract

Objective: The present study was undertaken to investigate the safe doses of *Pueraria tuberosa* water extract (PTWE) on different organs .**Methodology:** Haematoxylin and eosin staining was used to study the morphological alterations in heart, intestine, testis, adrenal gland and spleen. Followed the OECD guidelines 407 of repeated toxicity with respect to the selection of dose and days for different organs. The selected doses of PTWE were 250, 500, 1000 and 2000 mg/kg bw and the durations selected were 7, 14, 21 and 28 days. **Result**: According to the obtained results, we have found no any types of adverse alteration in cardiac fibers of the heart, size and shapes in crypts and villi of intestine, semeniferous tubules and spermatozoa count were normal in testis, all three zones of adrenal gland were normally identified and no any adverse sign of pulps in spleen was seen in all treated groups of PTWE **Conclusion**:There was no any types of adverse morphological alteration found in any organs. The drug PTWE are safe at 1000 mg/kg bw upto 28 days and 2000 mg/ kg bw upto 21 days respectively**.**

Keywords- Interstitial edema, Histopathological alteration, OECD, Organ toxicity, *Pueraria tuberosa*.

Introduction - In several countries, more than 60% of the population are dependent onherbal medicine for health care. However, Because of non-availability of safety parameters, many people are hesitant in using herbal medicine. **A**lthough it is well established that herbal medicines have therapeutic responses and they are already in clinical use. There are also other regions of the world (e.g. China, South Pacific, and India) with some problem related to herbal adverse drug reaction.

In this study, the partially purified water extract of tubers of *Peuraria tuberosa* (PTWE) has been used to assess its toxic effect on albino rats as per OECD guidelines. The powder of PT tubers is in clinical use in Ayurveda as health promotion medicine (Sherman et al., 2010) . In Hindi it is called as vidarikand and in English it is Indian kudzu. It belongs to the family Fabeacea (Prasain et al., 2012). Its major secondary metabolites include puerarin, tuberostan, genestein, daidzin, tuberosin, puerarin, pterocarpin puerarone (Maji et al., 2014b) (Rastogi et al., 2013)(Maji et al., 2014a) .In kudzu root puerarin is the most abundant (approx. 23%w/w) and its potent ability to cause various pharmacological effects (Lee et al., 2005) .

Recently past in our lab we have done both basis & Ayurvedic research on diabetes (Srivastava et al., 2018a) . Here we have found that PTWE act as antidiabetic herbal drug working through incretin signalling pathway (Srivastava et al., 2018b) .

Further regarding its mechanism of action towards its anti-diabetic and nephroprotective potential its role of inhibition of DPP4, MMP-9, PKC-beta, and caspase activation. The results have shown activation of SOD, catalase, BCL-2, nephrin (Shukla et al., 2016) (Srivastava et al., 2015)(Srivastava et al., 2017). However, for the 1st time we reported its action of GLP-1, GIP, TNF-alpha, IL-6 and HIF-1 in different experimental conditions of diabetes and kidney damage(Shukla et al., 2017) . These results are according to earlier reports related to the role of different pure phytochemicals, which are also found in PT tubers(Bebrevska et al., 2008)(Cai et al., 2011) .The pre-clinical toxicity with reference to the effect of test drugs on different organs is one of the mandatory criteria for commercial release of any drug. Now, after WTO agreement, the OECD guidelines are mandatory. In India, Drug and cosmetic act 1940, revised from time to time also indicates similar studies. In recent past,

there was some modification in this act. Here the Y schedule is relevant, which has been described below.

Drugs and Cosmetics (5th Amendment) Rules, 2017, Ministry of Health and Family Welfare (Department of Health and Family Welfare) number G.S.R. 44,(E), dated the 17th January, 2017, published in the Gazette of India, Extraordinary, Part II, Section 3, sub-section (i), dated the 17th January, 2017

Histopathology is a broad area of science. The histological examination is the golden standard for evaluating treatment related pathological changes in tissue and organs (OECD. Organisation for Economic Co-operation and Development, 1995) .

The contribution of the pathological study should add information to the clinical data and should match new sensitive diagnostic markers.

It is very clear, reliable parameter to study the effectiveness/toxicity of drugs in animal tissue. Necrosis means leucocytic infiltration of cells. The best and simple methods used for the study of necrosis or any morphological alteration in tissue known as heamatoxylin and eosin by light microscopy. Study of safety and toxicity is the main parameter used before experimental study. The toxicity study conduct by OECD 407 guidelines. According to these guidelines, whole necropsy of all organs and histopathology of follow organs such as liver, kidney, heart, intestine, brain, thymus, testis, adrenal gland, pancreas and spleen is necessary (Pandey et al., 2018a). The herbal tablets of PTWE were prepared in our lab by a wet granulation method for clinical study (Pandey et al., 2018b).

Plant *Pueraria tuberosa* has shown many pharmacological activities in rats, which may be extrapolated to a different organ system.

On cardiovascular system. The puerarin, a flavanoid found in PT tubers has shown to exhibit hypo-cholesterolemic response (Yan et al., 2006) , and cardio-protective activity via sodium and L type calcium channel in guinea pig and rat (Zhang et al., 2013). The extract of PT tubers has shown anti-hypertensive property (Ng et al., 2011) (Wong et al., 2011). Puerarin has shown a protective effect against myocardial ischemia and reperfusion Injury (Gao et al., 2007) (Zhang et al., 2011).

On excretory system. The PT extract has shown diuretic potential, consistent with descriptions in traditional Ay texts. Later in our laboratory has reported its nephtoprotective potential against cisplatin induced acute kidney damage(Nagwani and Tripathi, 2010) , and kidney damage induced by Arsenic (Rani et al., 2017). Further reports indicate protection against diabetes induced chronic kidney disease .Further the mechanism of action for action

of PT extract has also been reported to be through its antioxidant and antiapoptotic potential. It is also reported to activate the MMP-9 activity and enhanced expression of nephrin protein (Shukla et al., 2016) .

On liver. The extract of PT tubers has shown hepatotoxicity at higher doses. Although, other reports have shown that some isoflavone, isolated from the flowers of this plant has shown hepato-protection in human(Tsuchihashi et al., 2009) and also in CCl_4 induced toxicity in rats and tert-butyl hyperoxide induced liver injury (Xia et al., 2013).

On digestive system. *Pueraria tuberosa* was reported to improve intestinal permeability those induced by alcohol(Zhang et al., 2009) . Pueraria was reported protective effect of intestinal cells with used in case of hepatic injury(Lee et al., 2002) .

On reproductive system. The extract of pueraria tubers has been shown to improve angiogenesis(Chauhan et al., 2013) and fertility potential in male as well as in females for enhancing lactogogue potential (Sherman et al., 2010) . The extract of pt and its isoflavenoids which act on such given types of following mechanism such as estrogen receptor, motility, arrometase recepter and chromatin condensation was being reported (Gray et al., 2015).

On Immunomodulatory system. The spleen act as filter for blood part of the immune system**.** The compounds isolated from tubers of *Pueraria tuberosa* have shown immunomodulatory and haematopoeis potential. The immunomodulatory effect of *Pueraria tuberosa* described earlier with use standard isoflavopuerarin such as, daidzein and genistein in swiss albino rat (Maji et al., 2014a) .

Objective –

Earlier, we reported the effect of PTWE in pre-clinical toxicity in rats as per OECD guideline 425 and 407 with special reference to changes in liver and kidney functions, necropsy of all other organs(Pandey et al., 2018a)(Calil Brondani et al., 2017). Here we are reporting its effect on other organs after oral treatment with different doses for different durations up to 28 days. The histological changes have been observed in Heart, Intestine, Testis, Adrenal Gland and spleen in rats of Charles foster strain. The purpose of this study was to assess the safest duration and PT extract dosage to rats organ's health, as it is reported to be health promoting in Ayurvedic texts.

Animal Design: In breed rats of Charls foster strain was purchased from the central animal house of our Institute (542/GO/ReBi/S/02/CPCSEA). The animals were acclimatized for 7

days in our laboratory condition and subjected to anti-protozoa treatment by giving metronidazole antibiotics. Finally the animals were randomly divided into five groups of 6 animals in each. The experimental protocol was approved by the institutional ethical committee (Dean/2017/CAEC/721).

Experimental design

The selection of dose and time of drug treatment was followed as per earlier studies, and the experiment was carried out as per OECD guidelines 407(Calil Brondani et al., 2017) . The dose was prepared by dissolving PTWE in water with gum acacia and different dose (250 mg/kgbw, 500 mg/kgbw, 1000 mg/kgbw and 2000 mg/kgbw) were orally given for 28 days, to each rat of the respective groups, in the morning time. Weekly assessments of body weight and diet intake were recorded. At the end, the animals were sacrificed by anasthetizing the rats by intra-peritoneal injection of pentobarbitone sodium (45mg/Kgbw). The blood was collected and within 5 minutes of death, all the required organs were dissected out. The attached fat and other undesired attached tissues were cleaned out and finally the organs were dried on the blotting paper and weighed on electronic pan balance. The chosen organs were heart, intestine, testis, adrenal gland and spleen, which were finally fixed in formalin for histo-pathological study. The animals of different groups were sacrificed at different time intervals of 7, 14, 21 and 28 days. The sample-tissue of the group having a dose of 2000mg/kgbw could not be collected at 28^{th} day because all animals of this group died . Though the post mortem was done and tissues were collected. Most of them showed a high degree of liver toxicity, as already reported in our preclinical toxicity study (Pandey et al., 2018a).

Histopathology. At The end of the experiment animals were sacrificed by giving pentobarbitone with euthanasia. Organs such as heart, spleen, testis, intestine, and adrenal gland was isolated and trimmed of excess fat in each group of animals, fixed in formalin. The standard protocol of making micro sections and staining was adopted(Troyer, 2008) . The fixed tissues were taken out of the fixative, washed properly and small sections were processed for dehydration procedure and finally embedded in the molten paraffin wax in a given orientation. After solidification, the blocks were trimmed, mounted on microtome (medimeas/mrm-1120 A) and thin sections of 5-6 µm thickness were cut and placed on slides, then coated with albumin and finally subjected to a process of dewaxing, dehydration and staining with hematoxylin and eosin (H &E) staining. Finally the sections were mounted

under a cover slip and sealed with Dopex. The T.S. of all the organs were examined and image were taken with binocular fluorescent microscope (Nikon Eclipse 50i Japan), fitted with a digital camera. Histological analysis was done to further confirm the alteration in cell structure of the organs. All the slides were randomly photographed in 10 view fields. The measurements of captured photographs were done by using software NIS Elements Basic research.

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Result–. Histological analysis was done by hematoxylin & eosin staining to further confirm the alteration in cell structure of the organs. After visualization of slides the result was analyzed for the organs, heart, intestine , testis, adrenal gland and spleen in normal rat as well as rat treated with different doses of PTWE i.e; 250 mg/kgbw ,500 mg/kgbw,1000 mg/kgbw and 2000mg/kgbw at 7, 14, 21 and 28 days respectively, The gross necropsy of above organ was found to be normal as earlier reported(Pandey et al., 2018a).

Normal Rat

Morphological expression in normal tissue. The heart tissue section has normal intactcardiac muscle myofibrils and intercalated disc Fig 1(A) . In the intestinal tissue, glands,mucosa, sub-mucosa, serosa, muscularis externa and villi fig 1(B). Spermatogonia are outer side , spermatozoa,spermatids and follicles is found in inner side of testis fig 1(C).in the section of adrenal gland shows an outermost portion is the cortex and inner section is medulla (fig 1D). in the splenic section morphological expression is shown by Red and white pulp (fig 1E).

Effects of PTWE on different organs at doses 250 500, 1000 and 2000 mg/kgbw **upto 7,14,21 and 28 days on heart, intestine, testis , adrenal gland and spleen.**

1- **Heart.** The T.S. of heart did not show any change in the extract treated group for all tested doses up to 21 days. The sections showed intact cardiac muscle myofibrils and intercalated disc **Fig1.** The treatment for 21 days with 2000mg/kgbw also did not show any cellular damage or focal necrosis of cardiac muscle fibers. No interstitial edema or swelling was also found (**fig 2)**

2- **Intestine**.The intestinal part, which was selected for study was 10 cm distal to the duodenum. Here also no significant change was observed in the PTWE treated rats. The T.S. pictures were similar to normal rat intestinal T.S. The serosa and mucous membranes were normal. The number and size of Vili were also normal. The glands, found in the lower portion of intestinal wall were also well shaped (**fig 3).**

3 – Testis. Histopathological examination of the testis shows normal histological structure of active mature, functioning seminiferous tubules associated with complete spermatogenic series are shown in fig 4 and fig 1. No edema and expansion of interstitial were founded b/w seminiferous tubules. The cells of spermatozoa, spermatogonia was found in fully mature condition in all sections of tissue of doses upto **2000** mg/kgbw for 21 days . There were no congestion and dilation was found in blood vessels . Our results are contrary to the literature, showing its anti-infertility and spermatogenic activating potential, at least in normal rats. It may be effective in patients, already having a defective physiology (**fig 4**).

4- **Adrenal gland.** The cellular appearance of the cells located in the zona glomerulosa, zona fasciculata, zona reticularis and adrenal medulla was intact in all tissue sections. No any types of morphological dissimilarity found in the cortex region of the adrenal gland because we know that adrenal gland necrosis found more frequently In the cortex (especially zona fasiculata and reticularis) then medulla (Rosol et al., 2001) .

There is no amorphous debris was found in focal areas of zona fasiculate of the higher dose of PTWE i.e; for 100 and 2000 mg/kgbw upto 28 and 21 days **(fig 5).**

5- Spleen. The T.S. of the spleen showed the normal architecture in red pulp and white pulp region in all the tested doses of PTWE. The red pulp region consists of red blood cells, lymphocytes and plasma cells, whereas the white pulp region contains T lymphocytes and B lymphocytes. The degree of haematopoiesis, observed in the red pulp region was also normal**(fig 6)**.

Discussion. The pathological study must be performed at the appropriate technical and interpretative levels to confirm, extend and improve information useful for the clinical understanding of the events. As compared to growing the number of herbal drug users around the globe, there is a lack of scientific data on the safety profile of herbal products(Saad et al., 2006) , therefore the safety of these product has become an important issue(World Health Organization, 2004) . In this experiment we have conduct OECD guidelines 407 and found that histopathological examination of the vital organ did not reveal any morphological changes after oral administration of Pueraria tuberosa for 28 days at the dose level of 250 mg/kgbw, 500 mg/kgbw, 1000 mg/kgbw and 2000 mg/kgbw. The all morphological alterations in the following tissues were examined by the histopatho-physiological study.

Heart. In the present scenario, cardiovascular diseases, particularly, become a worldwide health problem affecting all economic groups of the society.

Oxygen free radicals are known to generate during periods of ischemia followed by reperfusion. Heat shock proteins (HSP) play a critical role in maintaining cellular homeostasis and protecting cells against oxidative stress. Specially, HSPs of the 70 kDa family (i.e., HSP72) is important in developing tolerance to ischemia-reperfusion injury(Powers et al., 2001) . Tannins, flavonoids and glycosides have significant antioxidant properties, thus augments antioxidants and induction of HSP 72(Nieto et al., 1993) (huke I et al 1998) . In the previously reported isoflavonoids and glycosides are found in plant *Puerraia tuberosa* . PT is also reportedly known for its antioxidant property (Pandey et al., 2007). The major bioactive component of the PT is flavonoids known as as puerarin. On the basis of previous study of puerarin and ours present result, we can assume peurarine might be the possible component of the PT which protect the cardiovascular tissue via a different mechanism as puerarin lower the mRNA & protein level and its receptor APJ in one clip hypertension (Jin et al., 2009), and blocks TSP 1 expression in diabetic rats (Pan et al., 2009). In another way puerarin shown a protective effect in isoprenaline induced myocardial fibrotic mouse model via lowering the mRNA level in TGF- b1 and protein expression of NF-kB and

up-regulation of PPARα/ in myocardial tissue (Chen and Chan, 2009). Therefore, in present study ,we found that higher doses of PT morphologically doesn't show any necrotic sign in heart tissue, because it have more isoflavonoids .

Intestine.The Intestine is the main primary organ, responsible for food materials and drugs absorptions. The small intestine comprises 4 layers i.e;, mucosa, sub mucosa, muscularis externa and serosa . Mucosa consists of 3 layers, epithelium, lamina proporia and muscularis mucosa organized into villi and crypts (crypts of liberkuhn). Villi are finger like projection of the epithelium which contain blood and lymphatic vessels into the intestinal lumen(G. Hunter, MD et al., 2010). Intestinal mucositis is a common side effect of clinical chemotherapy for patients with cancer(Wadler et al., 1998) , and includes symptoms such as severe diarrhea and dehydration. The antimetabolite anticancer agent, 5-fluorouracil (5-FU), is widely used to treat several types of malignant tumors, it frequently causes intestinal mucositis. Mucosistic is morphologically characterized by the shortening of villus height and destruction of crypts in the small intestine. Previously the apoptosis are detected in intestinal crypts 24 hours after the first administration of 5-FU in mice .The up-regulation of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β was similarly observed within 24 h of 5-FU treatment. We also found that 5-FU-induced apoptosis in the crypt was dependent on the up-regulation of these cytokines(Kitahara et al., 2012)(Yasuda et al., 2013) . Those puerarine responsible for anti-inflammatory activities via inhibiting the level of IL – 8(Pang et al., 2012) and suppressed the protein or the mRNA expression of TNF – alpha , NF-kB, iNOS, TGF- B1 and MDA (Li et al., 2013)[51]. In our result, no significant adverse effect was found in different doses of *Pueraria tuberosa* on morphological dissimilarity in size and shapes of the villi and crypts of intestine since it was reported that PT have more isoflavonoids .

Testis.

Drug induced toxicity was found in the testis by cisplatin and metabolic disorder such high fat diet induced diabetic model in rats. Various types of isoflavones, phytoestrogens puerarin those may be responsible for the stimulation of androgenic activity has been reported in the roots of *Pueraria tuberosa* (Chauhan et al., 2011)(Singh Chauhan, 2012). Phytoestrogen like daidzein and genistein also affect neurobehavioral are antioestrogenic either an action in opposition to that of oestradiol and increase the level of LH, FSH, and testosterone via stimulation of gonadotrophin releasing hormones GnRh (Lee et al., 2002) (Patisaul, 2005) . A

previous research was showing the PT responsible for reproductive system by the different mechanism such as estrogen receptor , mottling and chromatin condensations. We know that two types of estogen receptor ER α and ER β , the isofalvone phytoestogen which possess more affinity to bind in ER β (Kuiper et al., 1998)(Hwang and Jeong, 2008) [55,56,]. Spermatozoa motion is an expression of cell vitality. Puerarin may be a dominant Ca^2 modulating factor those reported on spermatozoan alkinematics, suggests interference with Ca2+influx in the cell (Rago et al., 2007) . An another important mechanism is chromatin condensations (Bajpai and Doncel, 2003). Chromatin condensations are responsible for mammalian spermatogenesis. Kudzu and puerarin combination with p4 may synergistically interfere with NADPH oxidation was reported in chromatin condensation (Chapman and Michael, 2003) (Bennetts et al., 2008). In our results no any types of morphological dissimilarities were found in rats after 28 days treatment of PTWE .

Adrenal. Flavonoids like Daidzein and nobiletin those found in PT was earlier reported for less, but significant increase in catecholamine synthesis or secretion via activation of extracellular signal regulated protein kinases (ERKs) through the plasma membrane estrogen receptor(Liu et al., 2007) thus enhance the symaptho adrenal system. The adrenal gland is an endocrine gland producing adrenaline and steroids like cortisol, aldosterone and androgens. Structurally, it has two parts called the adrenal medulla (inner part) secreting catecholamines (adrenaline and nor adrenaline) and adrenal cortex (outer part) secreting steroidal hormones aldoestrone, cortisol and estrogen(H. and R.B., 2011)(Marieb and Hoehn, 2013). The function of cortical region is controlled by a pituitary hormone called ACTH and that of the medullar region by nervous secretions. The cortical region is further divided into three zones: the zona glomerulosa, the zona fasciculata and the zona reticularis. The zone fasiculata produced glucocorticoids types of steroid hormones. The histological changes in adrenal could be correlated to its function. It is already reported that the release of the corticosteroids cortisol and aldosterone can be stimulated through the sympatho-adrenal system, by mediation through chromaffin cells in a paracrine manner. (Endocrinology127: 900–906, 1990), but no such changes have been observed in our study, suggesting no any types of adverse effect of PTWE on these systems .

Spleen. The isoflavones puerarin, daidzein, and genistein are the most important constituents, those responsible for the immunomodulatory function (Sawale et al., 2013). The effect of s*Pueraria tuberosa* and its isoflavones on haematopoietic system as well as on the function

of T cells and neutrophills have been extensively correlated with immunomodulatory function.

Immunomodulatory potential was evaluated against sheep red blood cells of splenic tissue(Maji et al., 2014a) .The leukocytes including neutrophils, lymphocytes, monocytes, eosinophils, and basophils are responsible for immune response. In our experimental study histo-pathological investigation of spleen did not exhibit any abnormalities treated with a low or high dose of Pueraria extract. Also spleen appeared grossly colored and no finding of congestion was found in all treated tissues. The tissue is clearly differentiated into white pulp (WP) and the red pulp (RP). The architecture of the white pulp displayed normal rounded scattered follicles.

Conclusion. No any types of adverse alteration were founded in cardiac fibers in the heart, Size and shapes in crypts of intestine, semeniferous tubules and spermatozoa count were normal in testis. All three zones of adrenal gland were normally identified and no any adverse sign of the pulps was seen in all the groups treated with 2000mg /kgbw of PTWE upto 21 days and with 1000mg/kgbw of PTWE upto 28 days. The drugs were found to be safe upto 2000mg/kg bw treatment of PTWE after 21 day**.**

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CONFLICT OF INTEREST: All authors declare that ,they have no conflict of interest

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Figure legends –

Fig 1 – Histology of normal rats (A)Heart ,(B) Intestine , (C) Testis , (D) Adrenal glnad and (E) Spleen . Scale bar 100 µm .Magnification at 40 X. ID- Intercalated disc, MF- Myofibrils,G-Glands,M-Mucosa,SM-Sub mucosa, S-Serosa, ME-Muscularia externa, V-Villi, SG-Spermatogonia,SZ-Spermatozoa, SD-Spermatids, F-Follicles, AM-Adrenal medulla, AC- Adrenal cortex, ZR-Zona glomerulosa ,ZF-Zona fasciculata ,ZG- Zona reticularis ,R-Red pulp, W-white pulp.

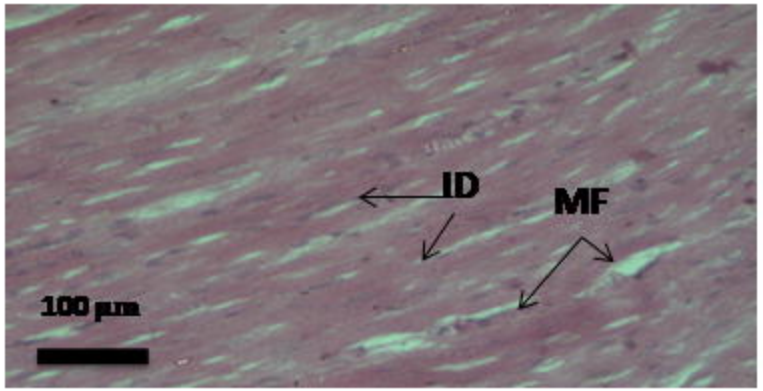
Fig 2- Histology of PTWE treated heart tissue at different time interval with variable dosage. Scale bar 100 µm. Magnification at 40 X.

Fig 3- Histology of PTWE treated Intestinal tissue at different time interval with variable dosage. Scale bar 100 µm. Magnification at 40 X.

Fig 4- Histology of PTWE treated Testis tissue at different time interval with variable dosage. Scale bar 100 µm. Magnification at 40 X.

Fig 5 Histology of PTWE treated Testis tissue at different time interval with variable dosage. Scale bar 100 µm. Magnification at 40 X .

Fig 6- Histology of PTWE treated Testis tissue at different time interval with variable dosage. Scale bar 100 µm. Magnification at 40 X **.s**



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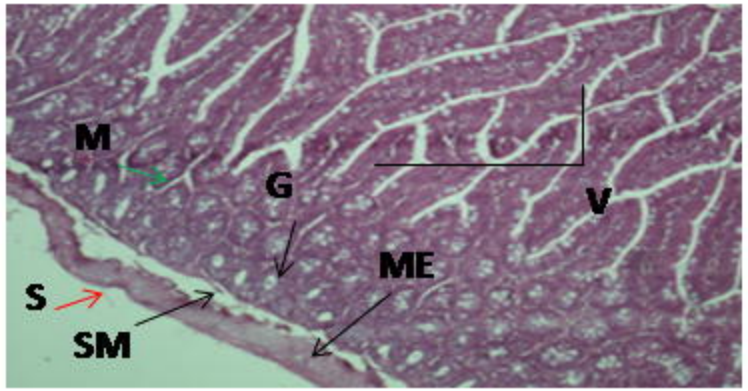

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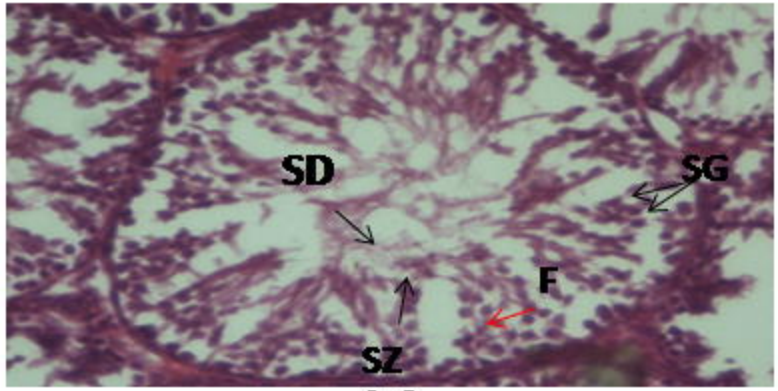

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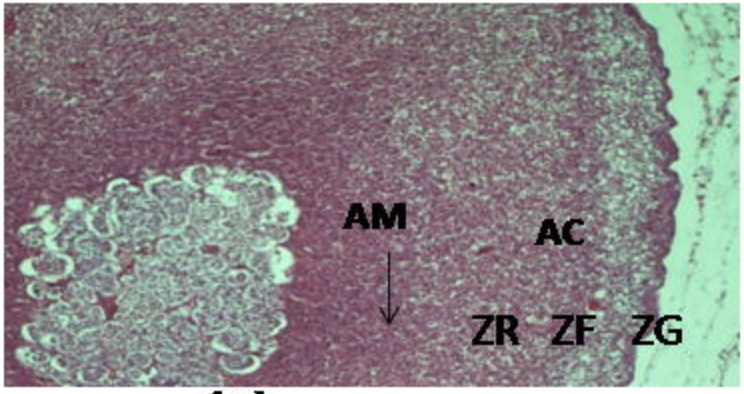

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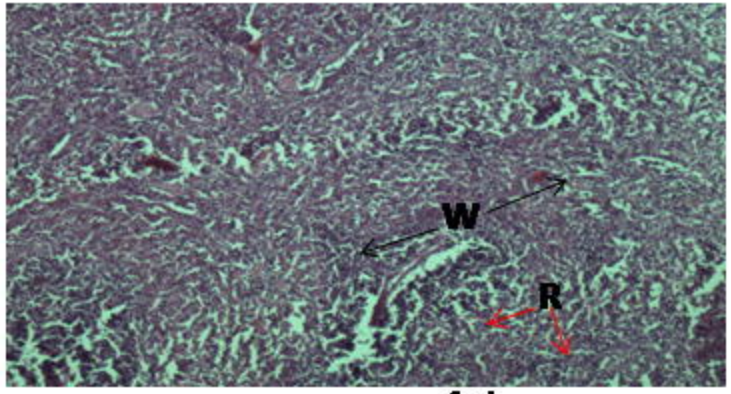

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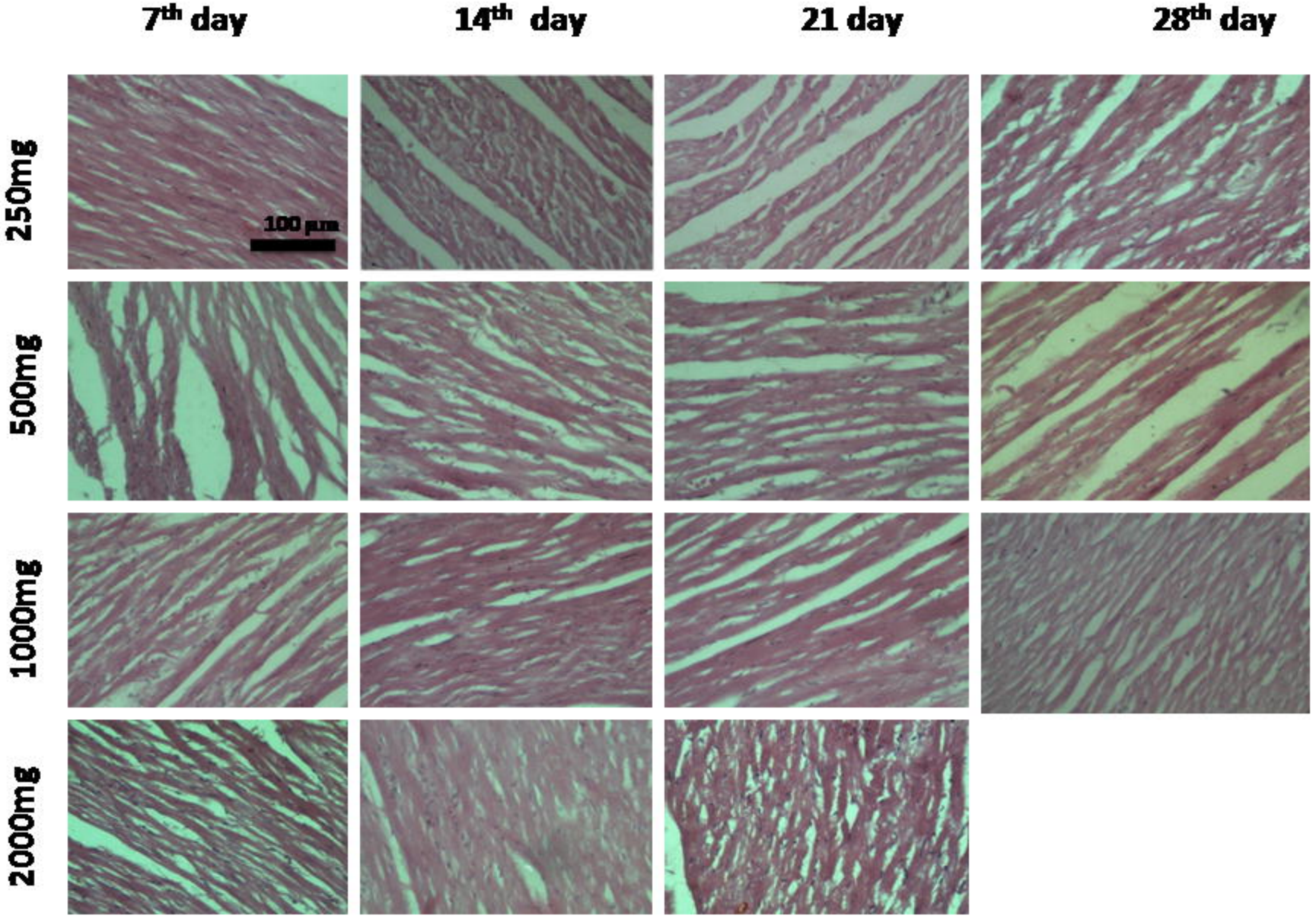
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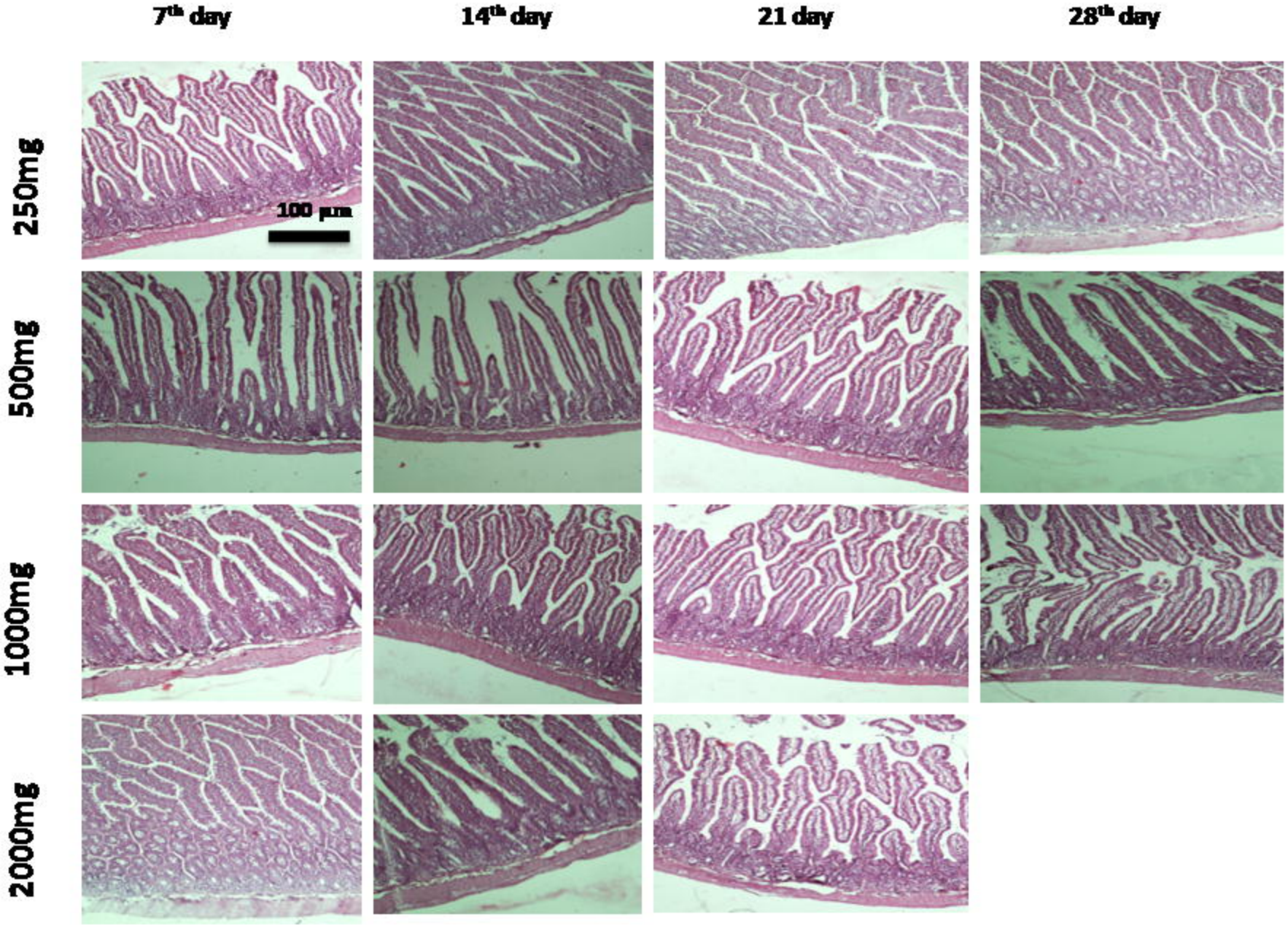
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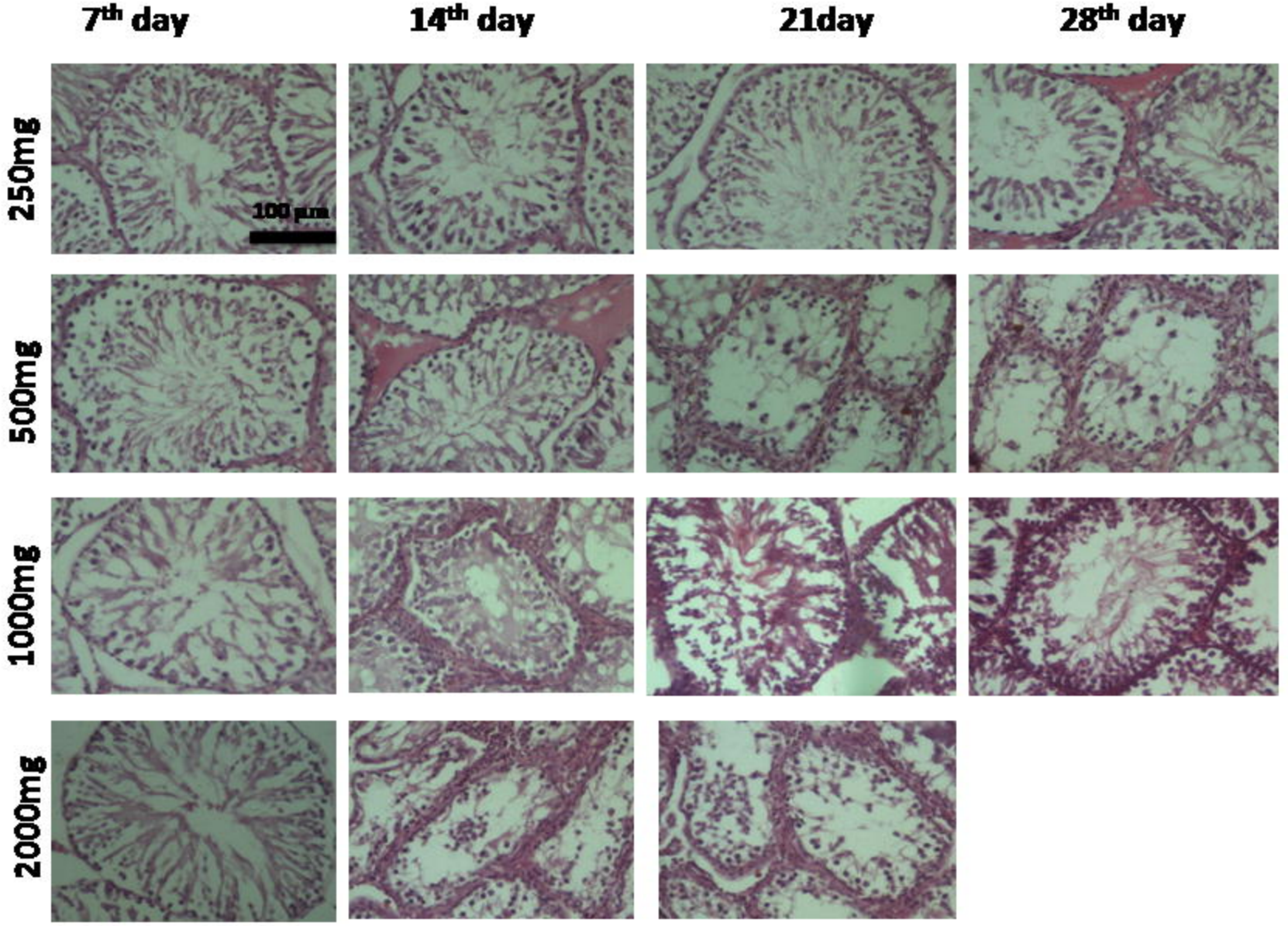
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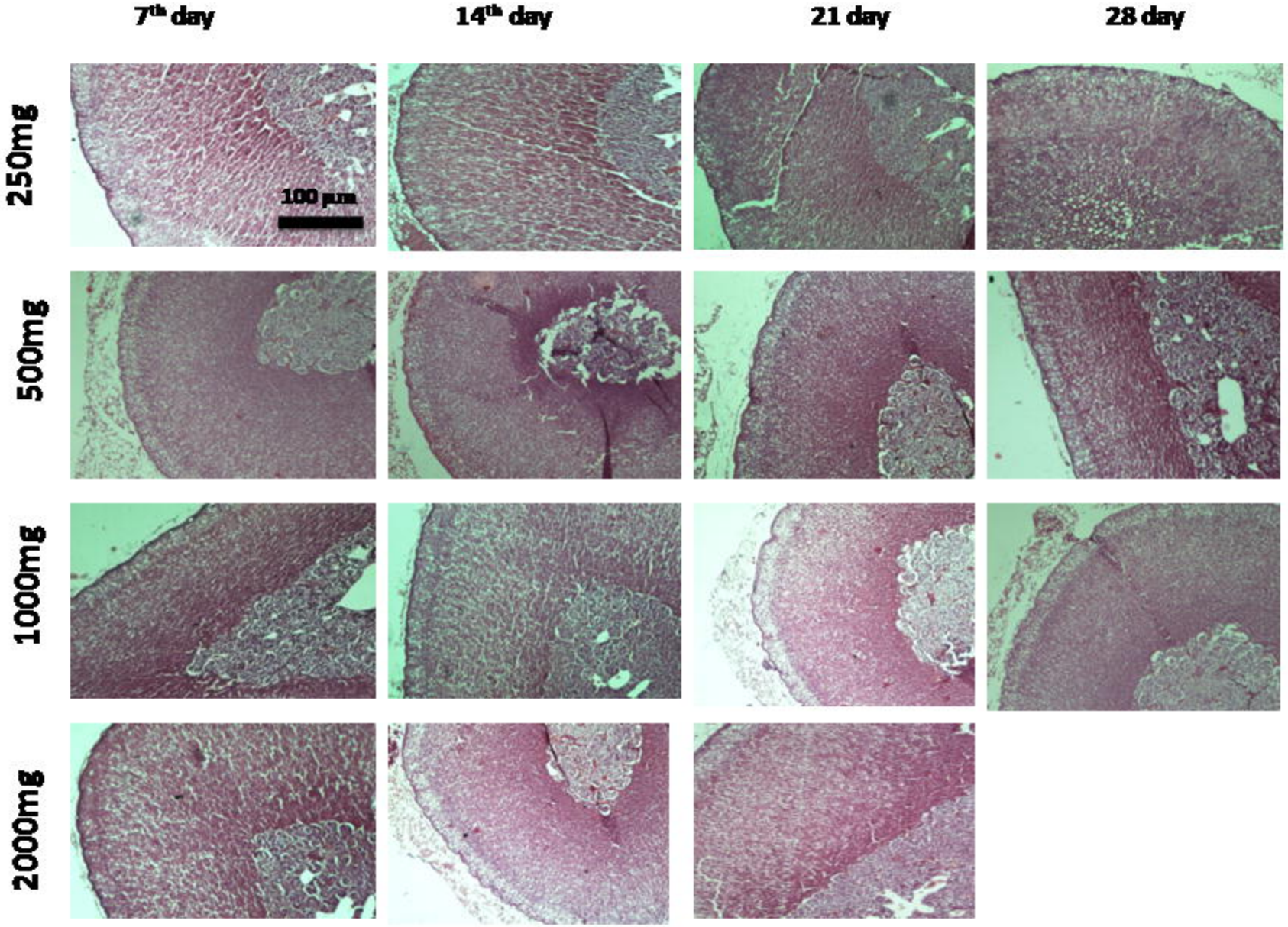
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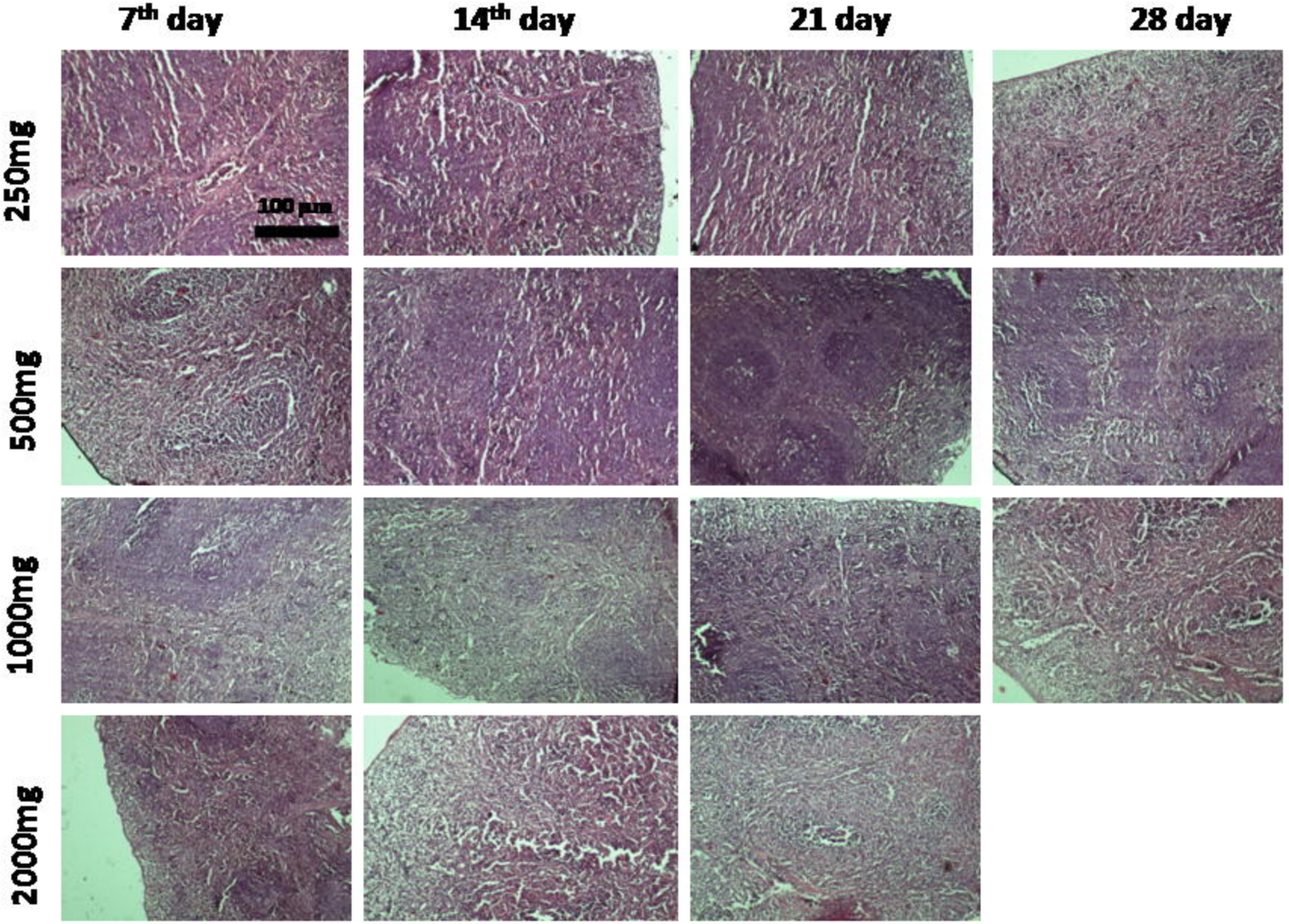
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