Original Article

A Stereological Study Concerning the Role of *Satureja Khuzestanica* Essential Oil in the Improvement of Mesangial Expansion in Uninephrectomized Diabetic Rats

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Abstract

Background and Aim: Mesangial expansion may lead to renal failure due to the stenosis or occlusion of glomerular capillary lumen in diabetic patients. Oxidative stress is the main cause of diabetic mesangial expansion. The information obtained from the accurate estimation of mesangium volume, glomerular capillary volume and mesangial cells number via stereological methods is more reliable than that of morphometric or semi-quantitative variables in diabetic nephropathy investigations.

Materials and Methods: Forty mature male rats were uninephrectomized and randomly divided into four groups as follows: group 1: the control group, group 2: diabetic without treatment, groups 3 and 4: diabetic treatment with SKEO 250 and 500 ppm in drinking water respectively. The treatment started after the induction of diabetes by alloxan. Treatment blood was sampled and the kidneys were fixed in formal saline solution after 8 weeks. Serum MDA was measured by thiobarbituric acid test. Kidney paraffin sections were prepared and stained via periodic acid Schiff (PAS) method. Mesangium volume, glomerular capillary volume and mesangial cells numbers were estimated via stereological methods. Data were analyzed via Mann Whitney U test at $p<0.05$.

Results: Diabetes increased serum MDA, mesangium volume, mesangial cells number, and at once decreased glomerular capillary volume in comparison to control group. Treatment by SKEO ameliorated these variables when compared with group 2 ($p<0.05$).

Conclusion: As an antioxidant agent, SKEO ameliorates diabetic mesangial expansion, glomerular capillary volume and mesangial cells numbers via inhibiting lipid peroxidation.

Keywords: *Satureja Khuzestanica*, diabetic nephropathy, mesangial expansion, stereology, glomerulosclerosis

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Introduction

Diabetic nephropathy (DN) is the main cause of end-stage renal disease (ESRD). Hyperglycemic condition induces the proliferation of mesangial cells and the excess of extracellular matrix production - mesangial matrix and glomerular basement membrane (GBM) thickening- (1-3) that named mesangial expansion in pathological term. Mesangial expansion leads to the narrowing and occlusion of glomerular capillary, the reduction of glomerular filtration rate (GFR) and finally renal failure. The molecular pathogenesis of diabetic nephropathy suggest that oxidative stress is the focal point of the mechanisms involved in DN. Hyperglycemia leads to the production of reactive oxygen species (ROS), especially superoxide anions. ROS induce renal injuries via cell membrane peroxidation, protein oxidation, renal vasoconstrictors, DNA damages, the rise and activation of NF-κβ, activation of PKC, AGEs formation and TGF-β induction (4, 5). Besides, there are many agents that induce oxidative stress in mesangial cells that include AgII, TGF-β, Oxidized LDL, AGEs, Aldosterone, amino acids and serotonin (6).

The review articles (7, 8) are recommended to be studied for further details. As a plant which is endemic to Iran, Satureja Khuzestanica (Marze Khouzestani in Persian) is widely distributed in the southern part of the country. The genus Satureja belongs to the family of Lamiaceae and Nepetoideae subfamily. It is famous in folk medicine for its therapeutic value as an analgesic and antiseptic plant. The essential oil of this plant showed antioxidant, antidiabetic and anti-inflammatory effect in experimental studies (9). In this study, Satureja Khuzestanica essential oil (SKEO) was used against mesangial expansion induced by diabetes and was studied stereologically.

Materials and Methods

Forty mature male Sprague–Dawley rats (180–200 g) were prepared from Pasteur Institute of Tehran. They were allowed to adapt themselves with the new location for one week. For the purpose of accelerating and achieving glomerular hypertrophy and intensive glomerulosclerosis, all rats were uninephrectomized from the left flank (9). The rats were randomly divided into four groups (10 per group) as follows: group 1: control nondiabetic, group 2: diabetic without treatment, groups 3 and 4: diabetic treatment with SKEO in different therapeutic doses.

Satureja extract was prepared from cultivated satureja Khuzestanica in Khorramabad as it was explained previously (9). Briefly, satureja extract was prepared from cultivated satureja Khuzestanica in Khorramabad (Lorestan Province, western Iran). Aerial parts of the plants were collected during flowering stage and were air-dried at ambient temperature in the shade. Aerial parts were hydro-distilled using a Clevenger apparatus for 4 h, giving yellow essential oil in 0.8% yield. The essential oil was dried over anhydrous sodium sulfate and then stored at 4°C.

Diabetes was induced after overnight fasting in the second, third and fourth groups by the injection of alloxan monohydrate (100 mg/kg) subcutaneously (10). The third and fourth groups received 250 or 500ppm SKEO in drinking water respectively. The treatment initiated on the first day of diabetes induction. Five days after the induction of diabetes, blood glucose was measured by glucometer, and since all rats had a blood glucose level of≥300 mg/dl (16.7 mmol/l); they were considered as diabetic (11). During the first five days after diabetes induction, 2–4 rats per group died because of alloxan toxicity. Rats were kept at 12/12 dark–light periods in 21±3°C temperature. All animals were allowed free access to food, water and libitum during experiment.

After an 8-weeks treatment, animals were anesthetized (Nesdonal 50 mg/kg ip), and then blood samples were prepared from heart and after clotting. Serum was, then, gathered by centrifugation (15 minute at 2000 g). Serum malondialdehyde was measured via thiobarbituric acid test. The rats were subsequently perfused from heart ventricle with saline and then formal saline (10%) for 10 min under anesthesia. After perfusion, the kidney were decapsulated, weighed and immersed in formal saline solution. After 48 h fixation, each kidney was cut into slices of approximately 1mm thickness. The kidneys slices were processed, and paraffin sections (5μ thickness)

were prepared. Two sections from each slice were selected (the first and the second sections) as section pairs. Sections were stained via periodic acid Schiff (PAS) method.

Stereological Study

The estimation of mesangium volume per kidney: Glomerular volume per kidney was estimated via point counting rule as explained in our research (9). The volume density of mesangium per glomerulus was estimated via point counting rule. Nine sections from each kidney (1 section per slice) were used. Microscopical image was transferred to a monitor via a camera-equipped microscope (Leica DFC camera). A coarse grid (points 6 mm apart) was superimposed on monitor images (figure 1) and points that fall on glomeruli were counted. Then a fine grid (points 3mm apart) was superimposed on the same image on monitor and points that fall on mesangium were counted (Figure 2).

At least 100 glomeruli per animal were studied. The volume density of mesangium per glomerule (Vv mes/glom) estimated via bellow formula (12).

Vv (mes/glom) = FPM / CPG. 4

FPM=point hitting mesangium CPG=points hitting glomeruli

4= coarse point area/fine point area

Total mesangium volume per kidney were estimated via this formula:

V mes/kid=Vv (mes/glom).V glom

Glomerular volume was estimated via this formula:

V glom/kid= Vv (glom/cortex).V cortex

V cortex= Vv (cortex /kid). V kid

The volume density of cortex per kidney [Vv (cortex/kid)] and the volume density of glomeruli/cortex Vv (glom/cortex) were also estimated via point counting rule from the kidney sections (9).

When the fine grid was superimposed on glomerular images on monitor, the points that fall on capillaries lumen were counted, and then the volume density of capillary per glomerulus was estimated through the formula

Vv (capill/glom) = FPC / CPG. 4

FPC=point hitting capillary CPG=points hitting glomeruli

4= coarse point area/fine point area

Finally the total volume of capillary per kidney was

estimated via multiplying of Vv (capill/glom) in V glom/kid.

The numerical density of mesangial cells per glomerulus was estimated via physical dissector. Briefly from each kidney slice, section pairs 5 micron apart (the first and second sections) were used. The microscopic glomerular image of the first section (reference) was projected on dissector frame 1(5cm×5cm on paper), and the image of the same glomerulus from the second section (look up) was projected on dissector frame 2 (5cm×5cm on paper) at the total magnification of 1200 in two projecting systems simultaneously. Mesangial cells were counted if they presented in frame 1 but not on frame 2 and did not hit with forbidden lines of dissector probe (lower and left lines of frame 1). At least 100-mesangial cells were counted per animal slides. The numerical density of mesangial cells per glomerulus (Nv (mes/glom) was estimated from the following formula (13).

Nv (mes/glom) = ΣQ.M^2/ΣP.a.d

ΣQ= number of counted mesangial cells; ΣP =sum of studied fields from reference sections; a = dissector frame area; d = dissector height (section thickness); M= final linear magnification.

Finally the total number of mesangial cells per kidney was estimated via multiplying of Nv (mes/glom) on glomerular volume per kidney.

The Animal Ethics Committee of the Lorestan University of Medical Sciences approved this study which is also in accordance with the National Health and Medical Research Council guidelines.

All values were represented as mean±S.E.M. The data were compared between groups by SPSS 13 software via Mann–Whitney U-test. The differences were considered significant at $p < 0.05$.
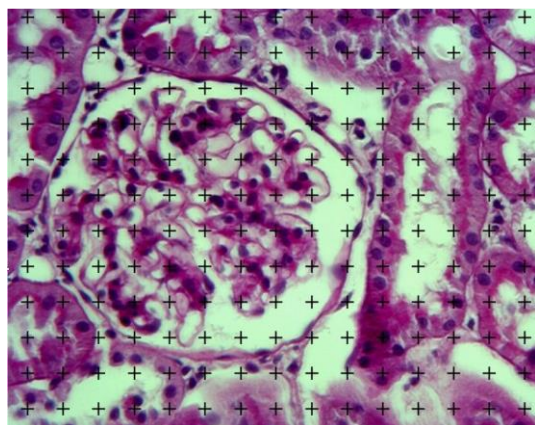
Ethics statement

Ethical approval has been received from the Ethical Committee of Lorestan University of Medical Sciences (animal scientific procedures act, approved by Iranian Ministry of Health and Medical Education.

Results and Discussion

Diabetes increased serum MDA concentration in

Figure 1. The coarse point grid (6 mm apart) was superimposed on micrograph and points that fall on glomeruli were counted. PAS staining, Mag 490.
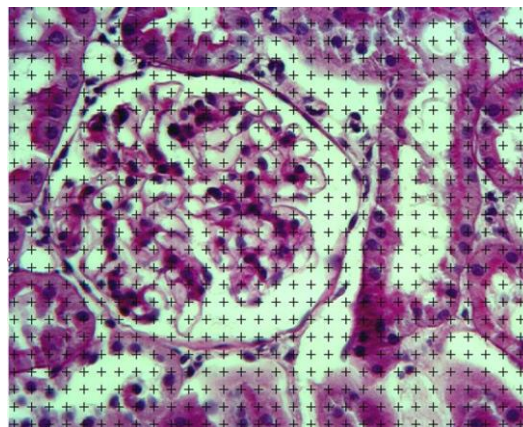
Figure 2. The fine point grid (3mm apart) was superimposed on micrograph and points that fall on mesangium or capillary lumen were counted. PAS staining, Mag 490.

Table 1: The Effect of Satureja Essential Oil on Serum MDA and Mesangium Volume in Alloxan Induced Diabetic Rats.

Experimental groups	Serum MDA (nmol/ml)	Mesangium volume(mm^3)/kidney
1:Control	0.51 ± 0.121	3.452 ± 0.46
2: Diabetic without treatment	1.757 ± 0.207*	8.722 ± 0.34*
3: Diabetic treated by 250ppm satureja extract	0.732 ± 0.134#	$5.854 \pm.0.27$*#
4: Diabetic treated by 500ppm satureja extract	0.628 ± 0.098#	5.02 ± 0.51*#

Values were represented as mean±S.E.M. * Significant change in comparison with control at $p < 0.05$. # Significant change in comparison with diabetic without treatment at $p < 0.05$.

Table 2: The Effect of Satureja Essential Oil on Glomerular Capillary Volume and Mesangial Cell Number in Alloxan Induced Diabetic Rats.

Experimental groups	Glomerular capillary volume (mm^3)	Mesangial cell number/kidney
1: Control	24.18 ± 0.91	1503504 ± 103582.3
2: Diabetic without treatment	16.4 ± 0.882*	2326687 ± 117710.5*
3: Diabetic treated by 250ppm satureja extract	19.32 ± 1.26*#	1765055 ± 129337.3#
4: Diabetic treated by 500ppm satureja extract	18.86 ± 0.57*#	1767565 ± 933825.4#

Values were represented as mean±S.E.M. * Significant change in comparison with control at $p < 0.05$. # Significant change in comparison with diabetic without treatment at $p < 0.05$.

group two compared to the control group, and SKEO inhibited the increase of serum MDA in treatment groups compared with group 2 (p<0.05). SKEO saved the Serum MDA at the same level of control group significantly (Table 1).

Mesangium volume significantly increased in group 2(diabetic without treatment) when compared to control animals. SKEO treatment significantly improved mesangial expansion in comparison to group 2 but treatments could not save mesangial volume at the same level as that of the control group (p<0.05) (Table 1).

Glomerular capillary volume was significantly increased by diabetes in group 2 when compared to the control group and was decreased significantly via the use of SKEO in group 3 and 4 (Table 2). Mesangial cell number/kidney significantly increased in diabetic group 2 in comparison with the control group, and SKEO treatment in group 3 and 4 improved mesangial cells number per kidney when compared with group 2 (p<0.05),(Table 2).

Mesangial cells are pericyte-like cells that are located intracapillary in glomerular capillary tuft. These cells maintain the integration of glomerular capillary,

production and participation in glomerular basement membrane turnover, regulating filtration surface area, intraglomerular blood volume and filtration pressure, secretion of growth factors such as PDGF, FGF, EGF, and CTGF, cleaning of GBM, and take up macromolecules entering into the matrix of the mesangium (14, 15).

The molecular pathogenesis of diabetic nephropathy suggest that oxidative stress is the focal point of mechanisms involved in DN. High glucose generates ROS via enzymatic and nonenzymatic reactions. The enzymatic reactions include NADPH oxidase, cyclooxygenase, lipoxygenase, xanthine oxidase, and myeloperoxidase. The nonenzymatic reaction include mitochondrial electron transport chain defects, advanced glycation end products (AGEs) generation, glucose autoxidation, Fenton reactions, and polyol (sorbitol) pathway(14,16). Meanwhile, ROS generate pathways, such as NADPH oxidase, and AGEs that are the most important of molecules involved in the pathogenesis of diabetic nephropathy (17, 18).

ROS induce renal injuries via cell membrane peroxidation, protein oxidation, renal vasoconstrictors, DNA damages, the increase and activation of NF-κβ, activation of PKC, AGEs formation and TGF-β induction (4, 5). There are many factors that induce oxidative stress in mesangial cells that include AgII, TGF-β, Oxidized LDL, AGEs, Aldosterone, Amino acids and serotonin (6). To obtain detailed information, see the review articles (7, 8). Moreover, ROS mediate damage in glomerular endothelial Layer, GBM (19) and induce damage to podocytes (20).

Cytokines, such as the transforming growth factor beta (TGF-beta), connective tissue growth factor (CTGF, epidermal growth factor (EGF), heparin bound EGF (HB-EGF) and insulin-like growth factor (IGF-1) directly affect mesangial cells in diabetic condition (21, 22).

Some transcription factors, such as the signal transducer activators of transcription (STATs), hypoxia-inducible factor (HIF1) alpha, Smad1, SRY-related HMG Box9 (SOX9), NF Kappa B, Wnt/beta catenin USF1 and USF2, Y-Box binding protein-1 and SP1, and sterol- responsive element-binding protein (SREBP)-1 are also involved in the activation of mesangial cells. These pathways lead to the

production of extreme amounts of matrix and glomerulosclerosis in diabetic cases (23-28).

Our results indicated the rise of serum MDA (lipid peroxidation marker) by diabetes. Furthermore, the use of SKEO reduced serum MDA similar to the use of other antioxidants by other investigators (11, 29).

There are many studies indicating the amelioration of mesangial expansion and glomerulosclerosis by different antioxidants, but all of these studies have done semi quantitatively or morphometrically (30-32), and variables such as mesangial area were measured or studied semi quantitatively by scoring of sclerosis (scoring, 0-4). In this research, mesangial volume (mm^3) was estimated by unbiased designed based stereological methods. Because of systematic uniform random sampling and design based stereological rules, stereological estimates are unbiased (33, 34). Our results showed that increased mesangial volume induced by diabetes could be ameliorated by the use of SKEO. No similar stereological research has been conducted that might compete with our study by the use of other antioxidant or other antidiabetic agents.

The decreased glomerular capillary volume per kidney (lumen of capillary in which blood circulates) in diabetic animals was improved by SKEO. Unlike our study, no research has estimated the glomerular capillary volume per kidney in diabetic nephropathy. Although the mesangial matrix volume and glomerular capillary volume were ameliorated by SKEO in diabetic rats, the treatments could not save volume of these variables at the same level as that of normal animals. Because of intracapillary location of mesangium, the increase of mesangial matrix and mesangial cell number reduced lumen of glomerular capillary, and then led to the decrease of filtration area and the reduction of glomerular filtration rate.

Our results indicated that diabetes could raise mesangial cells number per kidney and the use of SKEO could inhibit it and save the number of mesangial cell at the level of normal group significantly. Our results also showed that diabetic mesangial expansion is mainly is due to the rise of mesangial matrix production. Although the mesangial cell number was saved as normal level by the use of SKEO, mesangial matrix volume could not be saved at the level of normal animals. This study does not show molecular mechanisms pathways involved in

mesangial matrix production or mesangial cell proliferation. Hence, further molecular investigations are required.

Conclusion

As an antioxidant agent, SKEO ameliorates diabetic mesangial expansion, glomerular capillary volume and mesangial cells numbers via the inhibition of lipid peroxidation.

Acknowledgment

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Kriz W, Löwen J, Federico G, van den J, Gröne E, Gröne HJ. Accumulation of worn-out GBM material substantially contributes to mesangial matrix expansion in diabetic nephropathy. Am J Physiol Renal Physiol. 2017;312(6):1101-11.

2. Reidy K, Kang H.M, Hostetter T, Susztak K. Molecular mechanisms of diabetic kidney disease. J Clin Invest. 2014;124:2333-40.

3. Ostergaard M.V, Pinto V, Stevenson K, Worm J, Fink L.N, Coward R.J. DBA2J db/db mice are susceptible to early albuminuria and glomerulosclerosis that correlate with systemic insulin resistance. Am J Physiol Ren Physiol. 2017;312:312-21.

4. Satirapoj B. Review on Pathophysiology and Treatment of Diabetic Kidney Disease. J Med Assoc Thai. 2010;93:S228-41.

5-Kinaan M, Yau H, Martinez SQ, Kar P. Concepts in diabetic nephropathy: From pathology to treatment. J Ren Hepat Disord. 2017;1(2):10-24.

6. Abboud HE. Mesangial cell biology. Exp Cell Res. 2012;318:979-85.

7. Tavafi M. Diabetic nephropathy and antioxidants. J Nephropathology. 2013;2:20-7.

8. Zhanga L, Panga S, Denga B, Qiana L, Chena J, Zoub J, et al. High glucose induces renal mesangial cell proliferation and fibronectin expression through JNK/NF-κB/NADPH oxidase/ROS pathway, which is inhibited by resveratrol. Int J Biochem Cell Biol. 2012;44:629–38.

9. Tavafi M, Ahmadvand H, Tamjidipoor A, Delfan B, Khalatbari AR. Satureja khozestanica essential oil ameliorates progression of diabetic nephropathy in uninephrectomized diabetic rats. Tissue Cell. 2011;43(1):45-51.

10. Fernandes N.P, Lagishetty C.V, Panda V.S, Naik S.R. An experimental evaluation of the antidiabetic and antilipidemic properties of a standardized Momordica charantia fruit extract. BMC Complement. Altern Med. 2007;24(7):29-36.

11. Haidara MA, Mikhailidis DP, Rateb MA, Ahmed ZA, Yassin HZ, et al. Evaluation of the effect of oxidative stress and vitamin E supplementation on renal function in rat with streptozotocin-induced type 1 diabetes. J Diabet Complications.

2009;23(2):130-6.

12. Drummond K, Mauer M. The Early Natural History of Nephropathy in Type 1 Diabetes. Diabetes. 2002;51(5):1580-7

13. Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Miller A, Nielsen K. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS. 1988; 96:379-94.

14. Fakhruddin S, Alanazi W, Jackson KE.Diabetes-Induced Reactive Oxygen Species: Mechanism of Their Generation and Role in Renal Injury.J Diabetes Res. 2017;2017:8379327.

15. Schl¨ondorff D, Banas B. The mesangial cell revisited: no cell is an island. JASN. 2009;20:1179-87.

16. Johansen JS, Harris AK, Rychly DJ, Ergul A . Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. Cardiovascular Diabetology. 2005;4:5-12.

17. Satoh M, Fujimoto S, Haruna Y. NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. American Journal of Physiology: Renal Physiology. 2005;288:1144-52.

18. Cheng H, Wang H, Fan X, Paueksakon P, Harris RC. Improvement of endothelial nitric oxide synthase activity retards the progression of diabetic nephropathy in db/db mice. Kidney International. 2012;82:1176-83.

19. Nakagawa T, Tanabe K, Croker BP, Johnson RJ, Grant MB. Endothelial dysfunction as a potential contributor in diabetic nephropathy. Nature Reviews Nephrology. 2011;7:36–44.

20. Liapis H, Romagnani P, Anders HJ. New insights into the pathology of podocyte loss: mitotic catastrophe. American Journal of Pathology. 2013;183:1364-74.

21. Simonson MS. Phenotypic transitions and fibrosis in diabetic nephropathy, Kidney Int. 2007;71:846-54.

22. Daroux M, Prévost G, Maillard-Lefebvre H, Gaxatte C, D'Agati VD, et al. Advanced glycation end-products: implications for diabetic and non-diabetic nephropathies. Diabetes Metab. 2010;36:1–10.

23. Marrero MB, Banes-Berceli AK, Stern DM, Eaton DC. Role of the JAK/STAT signaling pathways in diabetic nephropathy, Am J Physiol. 2006;290:762–8.

24. Abe H, Matsubara T, Arai H. Role of Smad1 in diabetic nephropathy: molecular mechanisms and implications as a diagnostic marker. Histol Histopathol. 2011;26:531-41.

25. Haase V.H. The sweet side of HIF. Kidney Int. 2010;78:10-13.

26. Kishi S, Abe H, Akiyama H, Tominaga T, Murakami T, et al. SOX9 protein induces a chondrogenic phenotype of mesangial cells and contributes to advanced diabetic nephropathy. J Biol Chem. 2011;286:32162–69.

27. Wiggins JE, Patel SR, Shedden KA, Goyal M, Wharram BL, Martini S, Kretzler M, Wiggins RC. J Am Soc Nephrol. 2010;21:587–97.

28. Tominaga T, Abe H, Ueda O, Goto C, Nakahara K, et al. Activation of bone morphogenetic protein 4 signaling leads to glomerulosclerosis that mimics diabetic nephropathy. J Biol Chem. 2011;286:20109-16.

29. Winarska K, Malinska D, Szymanski K, Dudziak M, Bryla J. Lipoic acid ameliorates oxidative stress and renal injury in alloxan diabetic rabbits. Biochemie. 2008;90:450-9.

30. Fujita A, Sasaki H, Doi A, Okamoto K, Matsuno S, Furuta H, Nishi M, Nakao T, et al. Ferulic acid prevents pathological and functional abnormalities of the kidney in otsuka long-Evans Tokushima fatty diabetic rats . Diabet Res Clin Pract. 2008;79:11-17.

31.C hen X, Wu R, Kong Y, Yang Y, Gao Y, Sun D. Tanshinone IIA attenuates renal damage in STZ-induced diabetic rats via inhibiting oxidative stress and inflammation. Oncotarget. 2017;8(19):31915-22.

32. Yang Y, Chen G, Cheng X, Teng Z, Cai X, et al. Therapeutic

potential of digitoflavone on diabetic nephropathy: nuclear factor erythroid 2-related factor 2-dependent anti-oxidant and anti-inflammatory effect. Sci Rep. 2015;5:12377.

33. Howard, Vyvyan.; Reed, M. G. Unbiased Stereology : Three-dimensional Measurement in Microscopy Advanced Methods; 2nd. Taylor & Francis Routledge: 2005.

34. Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS. 1988;96(5):379-94.