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Histological Structure of the Plastinated Kidney Following Deplastination

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Abstract: Plastination is a laboratory process to obtain permanent dry tissue and organ sample. That can be used in the future for investigation and educational purposes. This methodology is based on dehydration and penetration of synthetic substances such as silicon into tissue. In this study, it was aimed to deplastinate previously plastinated kidneys in order to examine them under the light microscope. In this study, 14 sheep kidneys were used, seven samples of control and seven samples of plastination-deplastination (p / d) group. Kidneys in both control and p/d groups were fixed in 10% formalin. The samples in the control group were embedded in paraffin following routine tissue processing protocol. However, the samples in the p/d group were deplastinated in alcohol and methylbenzene and embedded into paraffin. 5 µm thick sections obtained from paraffin blocks were stained with hematoxylin and eosin (H&E), periodic acid-shiff (PAS) and then examined under the light microscope. Typical histological structures were observed in the control group. Small fragments were obtained as it was challenging to obtain sections from the P/d group blocks. Morphological structures were visible with some pseudo degenerations and wrong staining. This study is the first study that demonstrates alcohol and methylbenzene deplastination can be partially successful for evaluating plastinated kidney samples under a light microscope. However, we believe that the kidney may have limitations due to its wide parenchyma compared with literature conclusions. Nevertheless, more studies are required to develop the optimum protocols.
Keywords: *Histology, Kidney, Plastination, Sheep, Veterinary anatomy.*

Deplastinasyon Sonrası Plastine Böbreğin Histolojik Yapısı

Özet: Plastinasyon, gelecekte araştırma ve eğitim amaçlı kullanılabilecek kalıcı kuru doku ve organ örnekleri elde etmeye yönelik bir laboratuvar işlemidir. Kısaca bu metodoloji silikon gibi sentetik maddelerin dehidrasyona ve dokuya nüfuz etmesine dayanmaktadır. Bu çalışmada ışık mikroskobu altında incelemek için önceden plastine edilmiş böbrekleri deplastine etmeyi amaçladık. Bu çalışmada 7 örnek kontrol ve 7 örnek plastinasyon-deplastinasyon (p/d) grubu olmak üzere, 14 koyun böbreği kullanıldı. Hem kontrol hem de p/d gruplarındaki böbrekler %10 formalin içinde sabitlendi. Kontrol grubundaki örnekler rutin doku işleme protokolü izlenerek parafine gömüldü, ancak p/d grubundaki örnekler alkol ve metil benzen içinde deplastine edildikten sonra parafine gömüldü. Parafin bloklarından elde edilen 5 µm kalınlığındaki kesitler Hematoksilen ve eozin (H&E) ile boyanarak ardından periyodik asit-schiff (PAS) ile ışık mikroskobu altında incelendi. Kontrol grubunda normal histolojik yapılar gözlendi. P/d grup bloklarından kesitler elde etmek zor olduğu için küçük parçalar elde edildi. Morfolojik yapılarda bazı dejenarasyonlar gözlemlendi. Bu, plastine böbrek örneklerinin alkol ve metil benzen ile deplastinasyonunun ışık mikroskobu altında değerlendirilmesinde kısmen başarılı olabileceğini gösteren ilk çalışmadır. Ancak literatürdeki sonuçlarla karşılaştırıldığında böbreğin geniş parankiminden dolayı bazı kısıtlılıklarının olabileceğini düşünmekteyiz, ancak optimum protokolleri geliştirmek için daha fazla çalışmaya ihtiyaç vardır.
Anahtar Kelimeler: *Böbrek, Histoloji, Koyun, Plastinasyon, Veteriner anatomi. ,*

Introduction

Preservation of the body or tissue of the dead has taken humankind's attention from the very beginning of human history. Instinctively, throughout history, humankind aimed to find a way to embalm the remains of dead human and animal species. During the ancient Egyptian period, humankind used organic chemicals to preserve the dead body, but organic treatment during this process is obscure as there are no remaining records (Buckley et al., 2001). Even embalmed paleoparasitological remains were found in embalming rejects jars in Egypt in recent history (Bouchet et al., 2003). Nowadays, tissue and cell preservation are widely used in medicine and animal-related sciences to preserve cells or the total body of species for education, examination, and even medical use. For example, animal taxidermy is widely used to preserve the remains of the dead, mainly the skin of the dead animals.

In medicine, liquid nitrogen is widely used as a preservative to store and keep cells alive before medical treatments such as chemotherapy which affect fertility. Liquid nitrogen can be used in cell culture-related studies as well. Also, formaldehyde

is still one of the most used preservative chemicals for educational purposes, mainly in morphology-related lessons. However, long-term formaldehyde storage may lead to problems in tissue preservation, processing, and staining protocols for histological examinations (Rhodes, 2013). In recent years, it was also reported that formaldehyde might cause health problems such as upper respiratory and tract problems, even nasopharyngeal cancer, and leukemia (Lan et al., 2015; Zendehdel et al., 2016). If self-care instruments are not used, formaldehyde may also lead to eye and skin irritation. Because of the toxic properties of formaldehyde, the development or exploration of a safer tissue preservative method has been a title of discussion between morphologists for a long time. In 1977, Dr. Gunther von Hagens (1986) developed plastination, an organ or body preservation method. In this methodology, lipid and water of tissue are replaced with curable polymer chemicals such as epoxy, polyester, or silicone, and the plastinated tissues have natural-looking, but dry, odorless, durable, and harden, and requires minimum care during storage (Al-Ali et al., 2009; Bolintineanu et al., 2017; Steinke et al., 2008; Van Hagens, 1986). Compared to formaldehyde, the safer property of plastination may allow this methodology to be replaced with formaldehyde preservation mainly in anatomy laboratories, which can be applied to all soft tissue types (O'sullivan and Mitchell, 1995). On the other hand, deplastination is the opposite process of plastination, believing that the samples can be returned to their natural form. Thus, deplastination will allow the samples to be used in various examinations after long preservation at room temperature (Ravi and Bhat, 2011).

Studies aiming to find a way to examine plastinated materials under a light microscope are a very new-born research title, and these studies based on this research area are minimal. Besides all soft tissues appropriate for plastination, one of the most examined organs in this preservation methodology is the kidney (Pereira-Sampaia et al., 2011). Kidneys are beans-shaped organs located at the posterior part of the abdominal cavity, next to the columnae vertebralis. Functionally, the kidney is an organ that takes functions during the dischargement of filtrated waste material from the blood. Waste materials in the blood are filtered via the functional parenchyma of kidneys, the nephrons, and urine then discharged (Rouiller, 2014).

Even though there are limited data for deplastination of kidneys, this study aimed to investigate morphological structures in deplastinated kidneys following plastination. Furthermore, determine whether alcohol and methylbenzene deplastination are suitable for sheep kidneys for microscopic examination or not.

Materials and Methods

Study design: This study was performed with the permission of the Experimental Animals Local Ethics Committee in Dicle University with a 35582840-604-02 approval number. In this study, 14 freshly collected sheep kidneys from slaughterhouses were used, and the organ samples were randomly divided into control and plastination-deplastination (d/p) groups of seven samples in each. The volumes of the kidneys were measured with a caliper, and data were collected for statistical evaluation. For further laboratory steps, the control and d/p samples were fixed in 10% formaldehyde. Samples in the control group were subjected to routine histological tissue processing protocol, and the samples in the d/p group were subjected to plastination and deplastination processes explained below.

Silicone plastination of the kidneys: In this study, all of the silicone plastination protocols were performed at the Plastination Laboratory of the Department of Veterinary Anatomy, Faculty of Veterinary Medicine in Fırat University. The necessary dissection procedures were performed, and the excess connective and fatty tissues were removed. Kidneys were taken to the dehydration step after being fixed with 10% formalin solution for a week. Afterward, fluid of kidney tissue was replaced with acetone (99.5%) in an insulated steel tank that kept -25 °C in a deep freezer. At the third bath of the acetone dehydration, tissue infiltration of the acetone was reached above 90% concentration. The whole dehydration process took 15 days. The samples were also kept in acetone for five days at room temperature to remove fat from the tissue. Following acetone exposure, the kidneys were taken into the vacuum tank to perform forced impregnation. This enforcement was performed by impregnating the S10, activated with an S3 catalyst in a 1/100 ratio. Forced impregnation of S10 took six days at room temperature. Then the samples were kept in an S10 / S3 mixture outside the vacuum tank for a day and outside the silicone-catalyst mixture the following day. The samples were taken out from the vacuum tank and kept for discharge of the excess silicone, which took five days. At the last step, the gas curing and hardening process was performed with S6, and the plastination process was completed. The hardening step in the gas curing unit took three days. In brief, the silicone plastination of sheep kidneys took 36 days.

Tissue processing protocol and deplastination: Kidneys in the control group were fixed in a 10% formalin solution. After fixation, the kidneys were dissected into small pieces, and total fixation was achieved 24 hours after the beginning of the experiment. Fixed samples were washed under tap water overnight and dehydrated in increasing alcohol series. Following dehydration, samples were cleared in xylene, then subjected to paraffin infiltration and embedded into paraffin. The obtained paraffin blocks were stored at room temperature for further examination. For deplastination, previously plastinated kidneys in the p/d group were kept in absolute alcohol for 24 hours. Subsequently, depolymerization of plastination substances was carried out by incubating kidneys in methylbenzene for 48 hours. Tissue softening was checked regularly by punching a needle, and at the end of the period, total kidney samples were dissected into small pieces. The samples were embedded into paraffin blocks after routine histological tissue processing protocol. The paraffin blocks were stored at room temperature for further sectioning (Ramos et al., 2018).

Hematoxylin-Eosin and PAS staining protocol: Five µm thickening sections were taken from the paraffin blocks with a rotary microtome, and the sections were transferred to positive charged adhesive slides. Paraffin depolymerization was performed by incubating the sections at 56 °C for an hour. The sections were cleared in xylene for hematoxylin and eosin (H&E) and Periodic Acid-Schiff (PAS) staining used for morphological examination and basement membrane evaluation. The sections rehydrated in decreasing alcohol series and brought to distilled water. H&E stained sections were stained in hematoxylin for 8 minutes, and then the samples were washed in tap water for 5 minutes. The sections were stained in eosin for a minute, and samples were immersed through increasing alcohol series. At the last step, stained samples were cleared in xylene and mounted with entellan. PAS staining was performed with a ready-to-use kit (Bio-Optica, Milano-Italy, Cat. No: # 04-130802). All steps of the PAS staining were performed according to the manufacturer's instructions. Sections passed through increasing alcohol series after PAS staining protocol, cleared in xylene, and mounted with entellan. Both H&E and PAS stained samples were examined under a light microscope, and micrographs were captured by a ZEISS Axio microscope (Bancroft et al., 2013).

Statistical analysis: The samples' measured weight, width, and length data in control and d/p groups were evaluated statistically. Non-parametric Mann-Whitney U test was used as statistics. Results were expressed as mean ± standard deviation (SD).

Results

Anatomical Results: Both inner and outer anatomical shapes of plastinated kidneys were quite similar to their natural appearance and retained their previous morphological characteristics (Figure 1). Capsula fibrosis was observed with its standard structure. The structure of the capsula adiposa was also preserved during the plastination process in one of 5 samples. Color range difference between cortex renalis and medulla renalis was less evident in plastinated kidneys then deplastinated (Figures 1a, 1b, 1c). The malpighi pyramides and A. interlobaris were observed clearly in plastinated samples. When the kidney was divided into anteroposterior semi parts, it was observed that sinus renalis was following the hilus renalis. The hilus renalis, which can be observed as a fold in the margo medialis, and the ureter originating from this anatomical structure were clearly observed (Figure 1d). The difference for weight, wide and height of the control group significant in contrast to P/D group ($P < 0.01$). Graphical demonstration of the statistical analysis was shown in Figure 2.

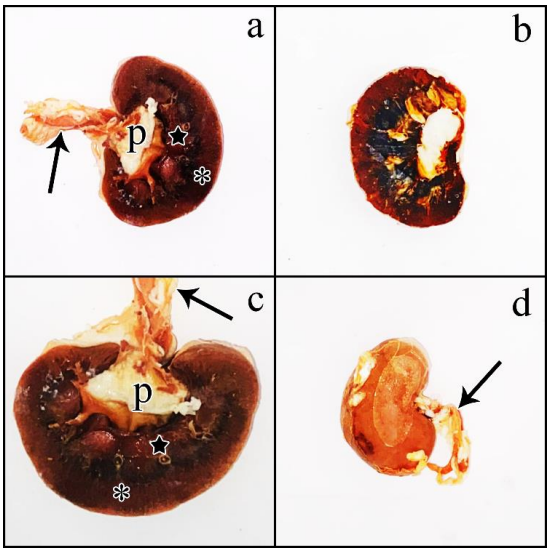

Figure 1. Inner (a,b,c) and outer (d) photographs of the plastinated kidneys. Renal cortex (*), medulla (star) pelvis renalis (p) and ureter (arrow) as shown

Histological result:

Hematoxylin and eosin results: Light microscopic micrographs of kidney sections in control and plastination-deplastination groups are shown in Figure 3. Sheep kidneys in the control group were found with typical histological structures. The proximal and distal convoluted tubules, cortical and juxtamedullary glomeruli were widespread within the renal pyramids. The renal vascular structures were normal morphology, and the arterioles at the

vascular pole of the renal corpuscles were normal as expected. The renal glomeruli were observed in fresh tissues as normal morphology with a glomerular capillary network, mesangial stroma,

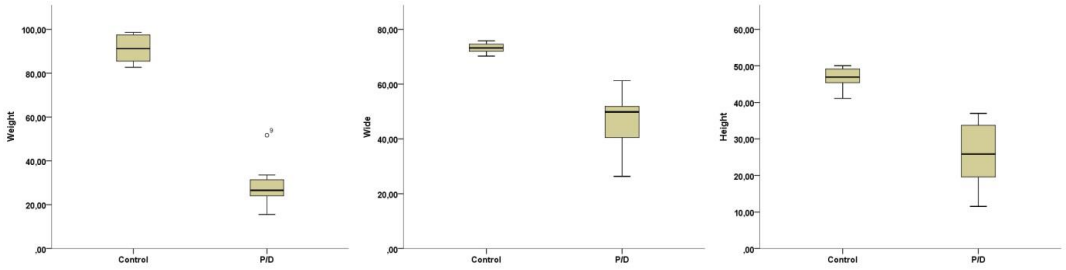

Figure 2. Boxplot results of statistical analysis for weight, wide and height. All evaluated parameters of groups were significantly different ($p<0.01$).

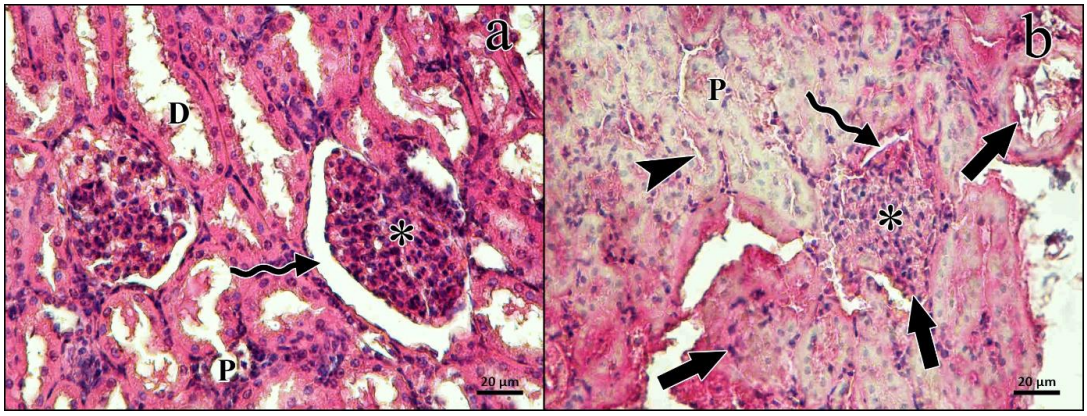

Figure 3. Micrographs of H&E stained control (a) and p/d (b) groups. Glomerulus with normal and shrunken morphology (asterix) in control and p/d groups. Proximal (P) and distal (D) convoluted tubules were easily distinguished in control group instead of incomprehensible morphology in p/d group. Bowman's space in control group (curved arrow) was normal but the space almost disappeared in p/d group. Cell nuclei in p/d group were stained hematoxylin (thick arrow). In p/d group, tubular lumen (arrow head) is observed as shrunken. Staining; H&E, Bar: 20 µm.

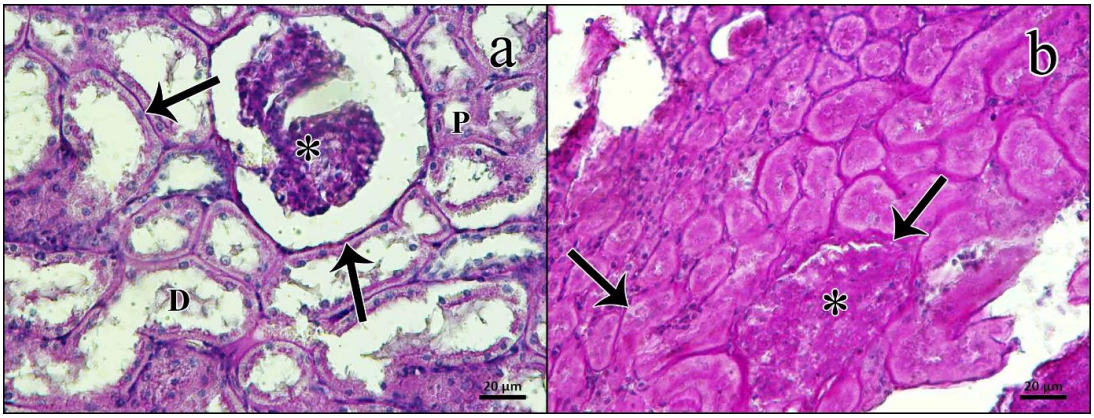

Figure 4. Micrographs of PAS stained control (a) and p/d (b) groups. Regular and normal shaped basement membranes (arrow) of glomerular basement membrane and tubular system in control group. In control group, glomerulus with normal morphology as observed normal glomerulary capillary network and mesangium (asterix). Pseudo-thickened and undulated basement membrane in p/d group's glomerular basement membrane and tubular system (arrow). Shrunken and pseudo-positivity of PAS in p/d group (asterix). Staining; PAS, Bar: 20 µm.

mesangial cells, and podocytes. Bowman's space between the glomeruli and the Bowman's capsule was observed in all glomerulus. The renal pyramids, loop of henles and collecting tubules were found normal, and the collecting tubules were longitudinal at the medullary level (Figure 3a). On the other hand, it was difficult to take sections from paraffin blocks of the p/d group because the tissue samples were pouring as dust while sectioning, but we could obtain small pieces of tissue samples from this

group. When the stained sections were evaluated under a light microscope, it was possible to observe cortical structures such as glomeruli, proximal and distal tubules. However, it was impossible to observe medullary structures due to the loss of this part during sectioning. Cortical structures were tightly packed, and Bowman's space was observed as almost disappeared (Figure 3b). In p/d samples, it was more difficult to distinguish the glomerular structures from each other than fresh kidneys. In H&E stained sections, glomerular, tubular, and capillary cell nuclei were visible in H&E stained sections. However, the eosin stain of cellular cytoplasm in this group was poor and total tissue was paler than the control group. Brush borders of proximal tubules of the p/d group were uncertain. Also, it was difficult to distinguish the proximal and distal tubules from each other without considering morphological differences.

PAS results: The control group's basement membranes of fresh tissues were regular in the PAS stained sections (Figure 4a). The basement membrane in the p/d group was PAS-positive, as observed in control group sections. However, it was thicker than the control group, probably due to the plastination-deplastination protocol. Additionally, the glomerular basement membrane of p/d group sections was unclear in contrast to the basement membrane of control group tubules. PAS positivity was also observed in the glomerulus of the kidney. However, the PAS positivity was not only observed in capillary basement membranes but also observed within the whole of the mesegial stroma. Although it only has to be observed at the basement membrane, the whole tissue sections of the p/d group were slightly pseudo positive for PAS. Kidney medulla samples were almost PAS negative because of deficiency of PAS reactions (Figure 4b).

Discussion and Conclusion

Scientific and technological developments have been shed light on tissue preservation methodologies until today. Today, it is highlighted that the plastinated samples can keep their natural morphological structure, but hardening is a disadvantage of this methodology when used for educational purposes (Bolintineanu et al., 2017; Reiderer, 2014). As pointed out in previous research studies (Ekim et al., 2017; Ottane et al., 2015), plastination provides dry, odorless, and durable specimens. Recent studies reported successful DNA extraction from plastinated tissues (Ottane, 2020). There are some articles on morphological and histological applicability of plastination of the urethra, esophageal muscles, and carpal tunnel (Fritsch et al., 2006; Sora and Gensen-Strab, 2005; Wang et al., 2007). Also, the kidney was reported with its suitability for plastination techniques (O'sullivan et al., 1995). However, in the literature review, no study did not investigate the microscopic evaluation of deplastinated kidneys. We observed a significantly reduced kidney size that was already reported for various plastinated organs in previous studies (Ameko et al., 2013; Rahul et al., 2020). As mentioned in the material and method, in this study, we aimed to investigate the microscopic structure of plastinated kidneys, which were deplastinated with alcohol and methylbenzene due to these chemicals were reported with successful results for deplastination of heart tissue previously (Baygeldi et al., 2020). In this respect, the result of this study of plastinated samples was consistent with the literature. The plastinated kidneys in the p/d group were strong with natural morphological structures and appropriate for educational use. We achieved partial success for histological examination in deplastinated kidneys with alcohol and methylbenzene, as Rahul et al. (2020) reported. They stated successful histological sectioning of the plastinated oral carcinoma with deplastination by 5% sodium methoxide dissolved in methanol (Rahul et al., 2020). We believe that difficulties in sectioning deplastinated samples in our study might result from dehydration during plastination or a large amount of parenchyma in the kidney instead of a muscular and more intact structure. We reported successful deplastination in the heart, which is almost occupied by heart muscle cells. Both of the reasons might be synergistically responsible for the limitations of deplastination in the kidney tissue. In this third possibility, we believe that S10 might not be impregnated in sufficient volume to keep microscopic structures intact for histological examination. Therefore, long-term shrinkage in dehydrated parenchyma might result from microscopic pseudo degenerations in deplastinated kidneys. According to a previous study (Brown et al., 2002), it was reported that 2 or 3 series of acetone baths should be performed depending on the samples for dehydration during plastination. Our study achieved over 90% concentration at the third bath.

Additionally, the ratio of the S3-S10 mixture used in forced impregnation step varies between previous studies (Jia-nan and Honq-jin, 2013; Jong and Henry, 2007). We found that the S3 catalyst at a ratio of 100/1 is sufficient to activate the S10 mixture at room temperature. In the gas curing step, we used the previously reported protocol Ravi and Bhat (2011) and observed it suitable for obtaining elastic samples. Although it may require effort to deplastinate large tissues, strong evidence demonstrates that deplastination with alcohol and

methylbenzene or other chemicals can allow histological examination of plastinated organs. In a previous study, Ravi and Bhat (2011) reported a slight delay for staining of deplastinated histological sections during staining protocol. The authors concluded as the tissues can be examined under light and electron microscope if the appropriate deplastination protocols are performed. In our study, we found the opportunity to capture images under the light microscope after deplastination with pale eosin staining. Therefore, it is possible to say that pale eosin staining in our results is consistent with the previous study of Ravi and Bhat (2011). It may be related to staining delay for deplastinated sections. Interestingly we did not observe any delay or pale staining in PAS stained sections in contrast to the H&E staining protocol. Overall, we observed slightly pseudo positivity at the whole tissue in PAS stained samples. Both Grondin et al. (1994) and our results demonstrate strong evidences that deplastinated tissue samples can be examined for histologically depending on the administered protocol.

In conclusion, the plastinated samples can be accessible when used for educational purposes or examination and do not require any care for transport of samples to elsewhere. Plastination allows any organ or tissue to be examined safely without the requirement of self-care instruments suggested to be used when samples were preserved in chemicals such as formaldehyde. On the other hand, we believe that plastination-deplastination may allow researchers to examine morphological structures of organs whenever required, even after long years. Our results demonstrated that plastinated kidneys were dry, clean, durable, odorless, and we observed partially successful results for histological examination after alcohol and methylbenzene deplastination. Therefore, we believe that improvements in plastination-deplastination methodology will open a new research area for morphologists, and more studies are required for protocol optimization.

Conflict of Interest

The authors stated that they did not have any real, potential or perceived conflict of interest

Ethical Approval

This study was performed with the permission of the Experimental Animals Local Ethics Committee in Dicle University with a 35582840-604-02 approval number.

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Author Contributions

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Data Collection and/or Processing: BCG
Analysis and/or Interpretation: BB, UŞ
Literature Review: UŞ
Posted By: BB, UŞ
Critical Review: BCG

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