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Design, Manufacture and Characterization of a Liver-Chip Model: A Platform for Disease Modeling and Toxicity Screening

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Abstract

BACKGROUND/AIMS: Drug research and development processes often fail in human clinical trials due to the inability of current *in vitro* cell culture and *in vivo* experimentation platforms to accurately predict toxicity and drug efficacy. There is an increasing need for human relevant systems which will enable studies on target tissue models prior to clinical trials. Drug-induced liver injury (DILI) is an important cause of acute liver failure. Species differences in liver toxicity and the limited predictability of traditional models represent a primary barrier to drug development for DILI. The aim of this study was to establish and characterize a dynamic microfluidic liver-chip with a physiological secretion function, continuous flow and compatibility with monitoring toxicity reactions.

MATERIALS AND METHODS: A biocompatible polymer-based prototype liver chip was designed using 3D stereolithography printing, CNC milling technology and molding. The chip with two microchannels, an upper channel for hepatocytes, and a lower microvascular channel for endothelium was produced by 3D bioprinting. The liver chips, pre-coated with collagen type I, were seeded with HepG2 cells and cell proliferation was monitored via microscopy. Toxicity was measured using a lactate dehydrogenase (LDH) assay. Albumin, alpha-fetoprotein (AFP), alanine transaminase (ALT) and aspartate aminotransferase (AST) secretion were also investigated.

RESULTS: LDH readings demonstrated that the designed microfluidic chip was non-toxic to human hepatocytes. Albumin, AFP, ALT and AST secretions were detected in cellular secretions at physiologically-relevant levels.

CONCLUSION: Overall, this study demonstrated the design and manufacture of a physiologically-relevant microfluidic liver chip model, which can be used in drug-monitoring and toxicity studies.

Keywords: Microfluidic system, 3D bioprinting, organ chip, drug monitoring, personalized medicine

INTRODUCTION

Drug development and disease simulation studies include both *in vitro* and *in vivo* stages. During these stages, multiple repetitions are performed in order to obtain the desired accurate results, leading to the consumption of large amounts of consumables and experimental animals. As a major drawback, incompatibilities between the two stages may occur. A disease model or a treatment which can be simulated at the cellular level can sometimes not be replicated in the animal model.[1] Therefore, scientists have sought models which can be studied both on a cellular level basis and closely represent the model organism. In the search for this model, organ-on-a-chip (OOAC) technology has

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recently come into use. OOAC systems contain engineered or natural human-relevant miniature tissues grown inside microfluidic chips. To better mimic human physiology, these chips are designed to control cell microenvironments and maintain tissue-specific functions.[2] It is a promising technology for effective drug discovery and drug screening for precision medicine. OOAC systems have made a significant advancement over the last 10 years. The OOAC was initiated with a lung chip, and has subsequently evolved into systems in which many different organs can be simulated.[3-7] The OOAC technology combines both cell culture and microfluidic systems. The most important distinguishing feature of these systems is that the cell culture medium is not static; it is stimulated with a continuous dynamic fluid flow and mechanical stimuli can be given if required. These mechanical stimuli can be in the form of stretching, which mimics the human organs, such as the simulation of the human respiratory movements via the stretching in a lung chip or peristalsis in a gut-chip. In addition, the dynamic flow of the culture medium in the OOAC eliminates the need for daily medium changes by the researchers, and the amount of consumables is considerably reduced due to the small size of the microfluidic channel.[8]

The liver is the largest internal organ of the human body, with its complex microarchitecture and function which plays a critical role in drug metabolism. Hepatotoxicity and drug-induced liver injury (DILI) are the main causes of drug failures.[9] Moreover, liver diseases are among the leading causes of death in the world, with new cases occurring each year. Although animal models have been traditionally used to investigate human drug metabolism and toxicity prior to clinical trials, rat and dog models have been reported to predict only 71% of the drug toxicities observed in humans.[10]

The aim of the current study was to design a three-dimensional (3D) microfluidic liver chip model which would reflect the characteristics of human liver physiology and pathophysiology; recapitulate the sinusoidal structure of the liver, maintain high cell viability and cellular phenotypes, and mimic natural liver functions. The designed liver chip would allow for the simulation of different disease phenotypes and aid in the development of safer and more efficient personalized treatments.

MATERIALS AND METHODS

Chip Design, Production and Assembly

For the mold design, a two-piece mold system which enabled the production of six chips was designed using the Fusion 360 (Autodesk Inc, USA) software. The production of the molds was accomplished both by using biocompatible resin in a Prusa SL-1 (Prusa Research, CZ) SLA 3D printer and CNC machining of aluminum. Quality control of the molds and canal diameter verification were performed via DinoLite (DinoLite Inc, USA) digital microscopy. The mold surfaces were cleaned with alcohol and distilled water. PDMS (Sylgard 184, Dowsil, USA) and a curing agent were mixed at a ratio of 10:1 and placed in a vacuum desiccator device for 30 minutes to remove air bubbles. This mixture was then poured into the molds and was incubated in an oven at 100 °C for 30 minutes. The two halves of the chip were then removed from the mold after cooldown and stored in dust-proof sealed boxes. Corning Transwell polyester membrane (10-micron thickness, 0.4-micron pore width) (Sigma-Aldrich, St. Louis, MO, USA) was used as the separating membrane between the chip channels. The membrane was cut from the Transwell wells with a scalpel to form strips which coincided with the middle channel of the chip. Two pieces separated from the molds and the previously prepared membrane in the form of upper part-membrane-lower part were applied to all surfaces with the Plasmatreat FG5001 (Plasmatreat GmbH) plasma activator device (IL, USA). As an alternative method, the surfaces of the upper part and the lower part were treated with uncured PDMS to adhere to each other and then cured in an oven at 60 °C for 12 hours. Subsequently, each chip was connected to the Syringe Two (Elveflow, FR) micro-perfusion pump and checked for watertightness. Cross-sectional controls of the first chips were also performed via DinoLite (DinoLite Inc, USA) digital microscopy.

Cell Culture

For cell culture, the HepG2 hepatocellular carcinoma cell line (catalog no: HB-8065, ATCC, Virginia, USA) was used. Vials containing 1 mL of frozen cell suspension at passage 10 were removed from a liquid nitrogen tank, thawed in a 37 °C water bath and added to 14 mL of cell culture medium (DMEM, 10% FBS, and 1% penicillin-streptomycin) (Invitrogen, USA). The cell suspension was centrifuged at 1,500 rpm for 5 minutes. After the centrifugation, the supernatant was gently decanted without disturbing the cell pellet. The cells were resuspended in 5 mL culture medium, and transferred into a 75 cm^2 flask (VWR, USA) containing 15 mL culture medium. After 24 hours of incubation at 37 °C with 5% CO_2, the medium was refreshed and placed in an incubator. The cells were observed daily under an Olympus IX53 inverted microscope and until 80% confluency was reached. Then, the culture medium was removed and the cells were washed with phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA). Three mL of 0.05% 1 x trypsin-EDTA solution (Thermo Fisher Scientific, MA, USA) were added to the culture dish and this was incubated at 37 °C for 10 minutes. After incubation, the cells were suspended in 10 mL of DMEM with 10% FBS and centrifuged at 1,500 rpm for 5 minutes. The cell pellet was resuspended in 10 mL of cell culture medium in a new cell culture dish and transferred to a 37 °C incubator with 5% CO_2. This procedure was repeated twice per week in order to ensure the continuity of the cell line.

Chip Loading with HepG2

Hydrogen peroxide gas plasma sterilization was performed for all of the chips manufactured. The chip channels were treated with 1 mg/mL Sulfo-SANPAH (sulfosuccinimidyl 6-(4'-azido-2' nitrophenylamino) hexanoate) solution (Thermo Fisher Scientific, MA, USA) with 50 µL in the upper channel and 30 µL in the lower channel. Activation was performed using 365 nm UV light for 20 minutes. Post activation, Sulfo-SANPAH was removed and the channels were washed first with 200 µL of sterile H_2O, and later with 200 µL of 70% ethyl alcohol. The channels were washed 3 times with 200 µL of sterile PBS in order to avoid ethyl alcohol residues. Subsequently, the upper and lower channels of the chips were coated with ~30 µL of 1 mg/mL collagen type I (Sigma-Aldrich, St. Louis, MO, USA) previously prepared with 0.25% acetic acid, and kept at 4 °C overnight. At the end of the incubation period, the collagen in the channels was removed, the channels were washed once with 200 µL of HepG2 culture medium and equilibrated for cell culture.

When 80% confluency was reached, the HepG2 cells were removed from the culture medium and washed with 5 mL of PBS. Then, 3 mL of 0.05% 1 x trypsin-EDTA solution was added to the culture dish and, after shaking gently, the culture dish was incubated at 37 °C for 10 minutes. After incubation, the cells were suspended in 10 mL of DMEM with 10% FBS and centrifuged at 1,500 rpm for 5 minutes. Then, the cells were resuspended in 300 µL of DMEM medium with 10% FBS and they

were stained with 10 µL of 0.4% trypan blue (Sigma) and counted on a Neubauer chamber. The cells were seeded at a concentration of $3x10^6$/mL cells (~30 µL) into the upper channel of a chip previously coated with type I collagen. In order for the cells to adhere to the chip, the chips were placed in an incubator at 37 °C with 5% CO_2 and incubated for 2 hours. After the incubation, the chips were examined with an inverted microscope. The chips were consequently connected to the syringe pump and the flow rate was set to 30 µL/hr. The input reservoir containing 10 mL culture medium and the empty waste output reservoir, chips and perfusion pump were placed into the incubator at 37 °C with 5% CO_2 for the duration of the experiment (Figure 1).

Chip Characterization

The HepG2 cells were examined with an inverted microscope at different incubation times including 6, 24, 36, 48 and 72 hours in order to monitor cell proliferation. Lactate dehydrogenase (LDH) measurements were performed using a colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA) to test for cellular cytotoxicity. The culture medium containing cell secretions collected in the output reservoir was used for the LDH assay. In addition, the albumin, alpha-fetoprotein (AFP), alanine transaminase (ALT) and aspartate aminotransferase (AST) levels in the output culture medium were measured using an ARCHITECT ci4100 Analyzer (Laboratories, Abbott Park, IL, USA) and compared with a DMEM culture medium without cells.

Statistical Analysis

No statistical analysis was required for this study.

RESULTS

Primarily, two mold designs were investigated in this study. It was determined that the first design caused an air bubble problem which prevented the flow of the culture medium, and therefore the study proceeded with design number two (Figure 2). The molds produced from resin by SLA 3D printing prevented complete curing of the PDMS thus leading to the adhesion of PDMS to the mold surface post curing. In order to solve this issue with the resin, the molds were subjected to both heat (60 °C) for 12 hours and a second UV (365 nm) treatment for 30 minutes. After this treatment, the PDMS was easily released from the mold. However, it was noted that the PDMS partially lost its transparency feature. However, PDMS chips which were cured at 100 °C for 30 minutes using an aluminum mold were easily released and showed proper transparency. Another failure was encountered during the air plasma activation of the chip halves to induce adhesion. The parts did not adhere after air plasma treatment, and therefore this method was discarded in this study. Instead, the chip halves were treated with a thin layer of uncured PDMS on the inner surfaces, a polyester membrane was placed in between, and the chips were assembled and cured at 60 °C for 12 hours. With this method, it was noticed that the two surfaces adhered to each other in a watertight manner (Figure 3).

The results indicate that, during the liver-chip culture, HepG2 cells were able to adhere to the upper channel of the liver chip at 1-hour post seeding as shown in Figure 4. Monolayer formation was examined under the microscope on the 3rd, 4th and 6th days post seeding (Figure 5). Culture medium containing cell secretions were collected in the output reservoir for LDH cell cytotoxicity measurements. The levels of LDH in the liver chips were found to be comparable with the DMEM culture medium without cells (Table 1). The cells, which formed a monolayer in the chip, were found to secrete albumin, ALT and AST. In addition, the AFP level in cells was found to be high and concordant with regenerating hepatic cellular physiology.

Figure 1. Incubator setup (chip, chip tray, perfusion pump, connectors, and output vials).

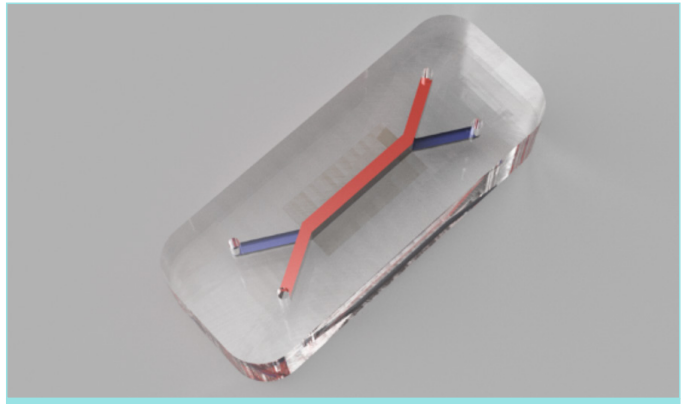

Figure 2. Chip design which was selected for this study (rendered in Autodesk Fusion360 [Autodesk Inc, USA)].

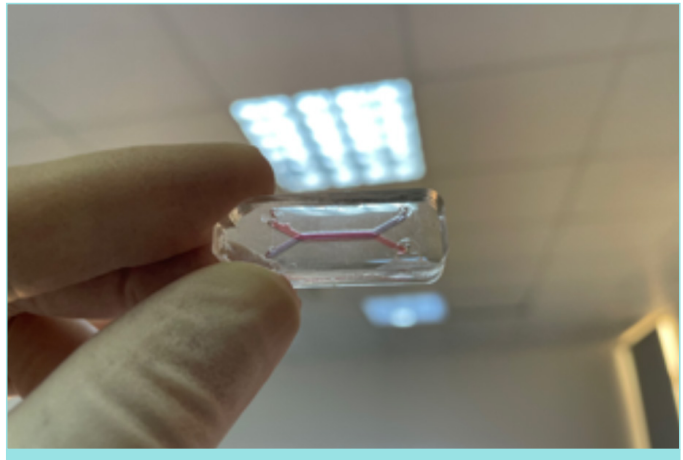

Figure 3. Assembled chip after water-tightness test (different dye colors indicate separate channels).

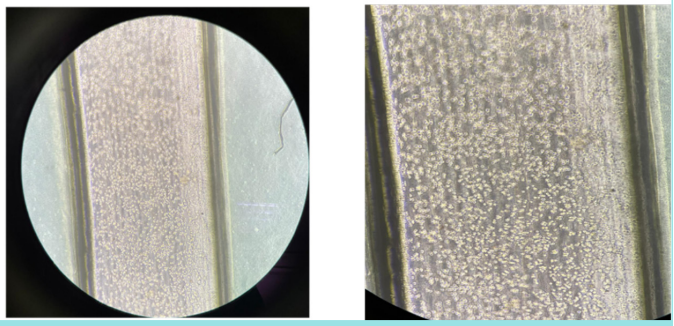

Figure 4. HepG2 cells attached to the chip upper channel 1-hour post seeding [(A) 10x magnification, (B) 20x magnification].

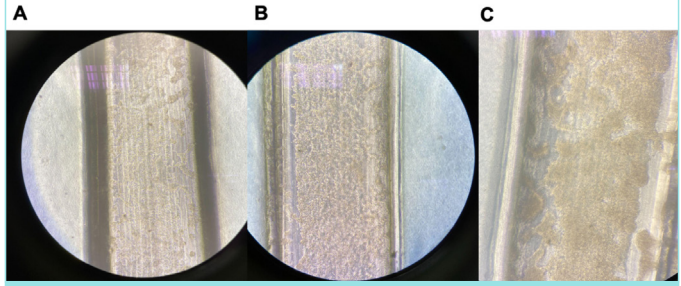

Figure 5. Proliferation of HepG2 cells on the liver chip and monolayer on (A) day 3, (B) day 4 and (C) day 6 (10x magnification).

Table 1. Comparison of the HepG2 liver chip and DMEM culture medium for cellular secretions

	Liver chip	DMEM without cells
LDH	45 U/L	37 U/L
Albumin	15 mg/L	7.41 mg/L
AFP	978 ng/mL	0
ALT	<6 U/L	ND
AST	3 U/L	ND

ND: Not detected, DMEM: Dulbecco's modified Eagle's medium, LDH: Lactate dehydrogenase, AFP: Alpha-fetoprotein, ALT: Alanine transaminase, AST: Aspartate aminotransferase.

DISCUSSION

Time and cost are two significant parameters for the R&D of new drugs. The development of a new drug takes about 12 to 15 years and costs about 800 million US dollars. In the R&D process, which consists of four phases, namely discovery and research, preclinical studies, clinical studies and treatment approval, 10 of approximately 10,000 compounds taken into animal testing reach clinical trials and only 1 is approved by the pharmaceutical agencies.[11] In this approach, only the preliminary physiological and pathological effects and toxicity results of the tested drug in tissue culture models can be observed. If the *in vitro* results are promising, the effects of the compounds developed are further investigated using animal models. However, these two models have significant disadvantages. For example, *in vitro* models cannot fully represent the complex cell-cell and cell-matrix interactions, and this incomplete interaction may lead to erroneous study results.[12] Drugs compounds shown to be effective *in vitro* may be ineffective during *in vivo* studies. Therefore, unexpected toxicity and low efficacy not detected during *in vitro* studies represent one of the most common and important causes of drug development failures. In addition, the highest rate of failure in drug development occurs in the second and third phases of clinical trials conducted in the final stages. For this reason, critical studies performed in the earlier stages significantly reduce costs and time spent in the process. The main reasons for the failure of new drug candidates can be attributed to the ineffectiveness of the existing *in vivo* and *in vitro* models, and the inability to detect serious side effects through these models.[13]

Although the current market size of OOAC technology is not as much as its potential, it was 7.5 million USD worldwide in 2016. According to some estimates, this market size will increase rapidly and may reach 163 million USD by 2023 with the completion of the existing R&D projects and an increase in the acceptance rates of organ chips in pharmacology.[14] Considering the use of the animals as well as the high costs, there is an increasing need for innovative alternative research processes which can achieve success in a shorter time, and which can be investigated using target tissue models before starting clinical studies, thus increasing the reliability of the R&D process. Recently developed bioengineered devices which mimic human organs or tissues have emerged as OOAC technology in the literature.[15] OOAC is an *in vitro* technique in which the mechanical and physiological responses of human tissues and organs are recreated and simulated by 3D microfluidic and other micro-scale supported structures.[16,17] Cell and tissue cultures made with standard cell culture flasks, petri dishes and well plates have many disadvantages such as being static, lacking extracellular matrix, and having deficiencies in modeling human tissue physiology, pathophysiology and microstructure. Microfluidic organ-chips enable 3D cell cultures in designed microchannels, and by better modeling complex tissues with multi-cell cultures, enable assays which cannot normally be performed with the existing experimental methods to be performed.[18] When previous studies on OOAC are examined, it can be observed that the importance of OOACs which can mimic the functions of various living organs, including the kidney, liver, lung, brain, skin, bone marrow and intestine has increased in recent years.[19-22] If the liver is taken as an example, the role of both new drug candidates and chemicals such as food additives in liver metabolism should be examined before human use. It is not possible to fully understand human liver tissue cells through animal models. Since *in vitro* cell lines proliferate on a single surface, they cannot be successfully simulated with liver tissue. With the OOAC design, it is possible to place hepatocytes, endothelium, Kupfer, stellate and iPSC cells into the microchannel in order to enable the physical simulation of the liver, or it is possible to create a spheroid, which is a 3D cell culture form from hepatocytes, and model 3D liver tissue within microchannels via these spheroids.[23,24]

With the current study, the first organ-chip study was established in our region, and although it was only a pilot organ-chip study, the results obtained were promising. In this study, it was observed microscopically that HepG2 cells continued their physiological development on the OOAC platform created, and the cells were observed to secrete proteins physiologically. Although HepG2 cells are hepatic carcinoma cells and are more disadvantageous compared to normal hepatocytes due to their proximity to the fetal liver, they represent a good model for proof-of-concept studies. During this study, significant potential advantages of microfluidic systems over conventional methods were observed. The most important of these is that the cells do not need intermittent media replacements due to the dynamic flow, and the positive effects of dynamic flow on cell physiology compared to static environment. The flow of dynamic DMEM or other culture media solutions in the chip channel has been proven to mimic the physiological blood or extracellular fluid flow of cells, and has also been shown to trigger growth. Although not included in the results, an increase in AFP secretion was observed within days in our study. It is also likely that risks of deterioration of the sterile environment will be reduced as this method requires less intervention. However, the most important advantage of microfluidic systems can be stated as being the use of very limited amounts of biomaterial. This allows a higher number of experiments to be performed with the same amount of material consumption compared to conventional methods.

In the context of the recent literature, a plethora of studies have been performed in order to demonstrate the superiority of microfluidic liver-on-a-chip systems over conventional cell culture methods in terms of their similarities to the physiology, mechanisms, and functionalities of liver cells in an *in vitro* environment. In particular, the use of primary hepatocytes obtained from liver biopsies or non-transplantable livers has been stated as being the gold standard for the development of human-relevant *in vitro* liver models, yet human induced pluripotent stem cells (hiPSCs), non-parenchymal cells and hepatic cell lines, which was the choice of cell source in the current study, are also being used as alternatives. In addition to monolayer cultures, matrix-free spheroids/organoids-on-chip, scaffold/hydrogel-based 3D liver OOACs and 3D liver OOACs using bioprinting are being considered as the emerging models for liver chips.[25] Furthermore, models such as the PhysioMimix liver-on-a-chip have recently been proposed and their utility has been shown to be effective for the exploration of multiple drug metabolism applications.[26] A hybrid polymer-based microfluidic platform, including cyclic olefin copolymer and PDMS, has also been developed for culturing hepatocytes and it has been demonstrated to retain the functionality and metabolic activity of perfusion culture as assessed by the secretion rates of albumin, urea, and cell viability visualization. This hybrid platform has been shown to produce comparable biomarker levels similar to published studies on other *in vitro* models.[27] More recently, a customizable microfluidic origami liver-on-a-chip model which simplifies the laboratory-scale fabrication of organ-on-chip models and hence speeds up chip development and optimization without reducing structural and functional features has been proposed.[28]

In the current study, the levels of albumin, AFP, ALT and AST values of HepG2 cells were also measured. Butterfield et al.[29] previously demonstrated that HepG2 cells cultured in 24-well plates produced AFP at 606 ng/mL/10^6 cells at 24 h. In our study, the amount of AFP secretion was found to be 978 ng/mL, which was comparably higher in the liver chip. Similarly, albumin secretion by HepG2 cells in tissue culture has been reported to be between 1.7-2 µg/mL, which is lower than the albumin values obtained in the liver chip (15 µg/mL).[30,31] Additionally, ALT and AST levels secreted by HepG2 cells in dishes or multi-well plates have been reported to be 3 IU/L and ~5 IU/L in the literature, respectively.[32] While these results are not superior to the cells in dishes, our results still demonstrated the presence of these secretions, which are important for physiological relevance.

Study Limitations

Under current conditions, experiments can be concurrently performed on four chips with a single channel or two chips with two channels. However, more equipment is required to perform experiments with higher numbers of chips. The liver chip secretions were only compared against cell culture medium without cells and not with secretions from cells grown without the chip. Another limitation of our study was the lack of immunostaining. This limitation did not pose a major problem as we biochemically and microscopically demonstrated that cell viability was preserved.

CONCLUSION

The liver chip model established in this study successfully demonstrated the viability of hepatocytes and physiologically-relevant secretions under constant flow. It is predicted that, in the near future, the OOAC models developed will replace experimentation on animal models and offer cheaper and faster potential new diagnosis and treatment methods. Therefore, future studies to improve and develop this technology are of paramount importance in order to advance this line of research.

MAIN POINTS

- There is an increasing demand for human-relevant micro-physiological systems which can accurately model and predict human diseases and aid in drug development processes.
- A microfluidic human liver-chip for modeling DILI was developed using 3D stereolithography printing, CNC milling technology and molding.
- The liver-chip model was non-toxic to human hepatocytes. The HepG2 cells secreted albumin, ALT and AST at physiologically-relevant levels. The AFP level in the cells was found to be high and concordant with regenerating hepatic cellular physiology.
- The model developed represents an advancement in drug-monitoring and toxicity studies.

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ETHICS

Ethics Committee Approval: Ethics committee form was not required. The study followed the principles of the Declaration of Helsinki.

Informed Consent: Informed consent form was not required.

Authorship Contributions

Surgical and Medical Practices: E.M., Concept: B.B., E.M., Design: B.B., E.M., Data Collection and/or Processing: B.B., E.M., Analysis and/or Interpretation: B.B., E.M., Literature Search: B.B., E.M., Writing: B.B., E.M.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study had received no financial support.

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