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Primary Murine Hepatocyte Isolation and Culture with GMP and Research-Grade Enzyme Blends

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1. Abstract

Hepatocyte transplantation for acute liver failure and metabolic liver disorders represent a potential alternative to traditional orthotopic liver transplantation (OLT). Additionally, cultured primary hepatocytes are an important tool for *in vitro* pharmacological studies. Advances in clinical hepatocyte transplantation and drug studies depend on the development of standardized isolation and culturing protocols as well as high-quality enzymes for liver tissue digestion. The isolation of primary hepatocytes for clinical applications must comply with current Good Manufacturing Practices (cGMP) regulations which may include the use of digestion enzymes produced to cGMP standards.

In this study, we compare VitaCyte® GMP-grade Collagenase MA in combination with BP Protease to Nordmark® Collagenase NB 6 GMP-Grade. Additionally, we compare non-GMP-grade Nordmark® Collagenase NB 4G Proved Grade to GMP-grade Nordmark® Collagenase NB 6 in an effort to identify a high-quality cost-effective alternative for hepatocyte isolation in non-clinical research applications. Our results indicated that Nordmark® NB 6 and VitaCyte® MA + BP blend produced hepatocytes with comparable pre-purification viable yields and viability. Similarly, non-GMP-grade Nordmark® NB 4G furnished primary hepatocytes with pre-purification yields and viabilities not significantly different from GMP-grade Nordmark® NB 6. This suggests that GMP-grade Nordmark® NB 6 is a viable alternative to GMP-grade VitaCyte® MA+BP enzyme blend for primary hepatocyte isolation, and that non-GMP-grade Nordmark® NB 4G is an affordable alternative for hepatocyte application in research. Additionally, GMP-grade VitaCyte® and Nordmark® are manufactured to cGMP standards and can therefore be utilized in the development of protocols intended for clinical application.

2. Introduction

The first successful whole-organ liver transplant occurred in 1967 [1]. Since then, advancements in surgical procedures and our understanding of immunological principles governing successful organ transplantations have opened the door for treatment of an array of acute and chronic liver diseases. And while transplants can save, and even improve the quality of individuals' lives, need consistently outpaces donor supply thereby limiting the number of transplant procedures possible each year [2]. Transplantation of high-quality fully-functioning hepatocytes represents an alternative treatment to traditional orthotopic liver transplantation (OLT). This approach offers two main benefits: it would help address supply issues since only a fraction of a whole liver is needed, and it is an overall safer procedure compared to OLT [2]. Additionally, cultured primary hepatocytes serve as an important *in vitro* model to study drug metabolism and toxicity, enzyme induction and inhibition, hepatocyte proliferation, and bioartificial liver systems [3,4,5]. These broad applications reinforce the need to develop cost-effective isolation techniques that yield high numbers of functionally viable hepatocytes.

Hepatocyte isolation techniques fall into two categories: mechanical dissociation and enzymatic digestion. Currently, enzymatic digestion is preferred over mechanical isolation as the rigorous preparation steps involved in the mechanical method such as dicing and shaking increase cell damage and reduce viable yields [6,7]. In contrast, the

enzymatic method allows the cells to better maintain their structural integrity and function post-isolation. The enzymatic approach was first introduced by Berry and Friend who reported that a continuous circulation of enzymes through the liver via the hepatic portal vein led to a 6-fold increase in cell yield [6,8]. Seglen further improved the enzymatic approach with a two-step method whereby the liver is first perfused with a Ca^{2+}-chelator followed by perfusion with an enzymatic solution [6,9]. Currently, the two-step collagenase perfusion method is the gold standard for hepatocyte isolation [10].

While many aspects of the two-step method are standardized, enzyme selection remains a key variable in determining overall hepatocyte cell yield and viability. It is also important to note that the manufacturing of hepatocytes for research and development purposes follows less stringent criteria compared to the production of hepatocytes intended for clinical application. The isolation of primary hepatocytes for clinical applications must comply with current Good Manufacturing Practices (cGMP) regulations which include the use of digestion enzymes produced to cGMP standards. These strictly-controlled and rigorously-monitored standards ensure high enzyme quality and lot-to-lot consistency thereby reducing variability in cell isolation outcomes [11].

VitaCyte® Collagenase MA represents a mixture of purified Collagenase Class I (C1) and Class II (C2) derived from *Clostridium histolyticum* and is combined with purified neutral BP Protease derived from *Bacillus polymyxa* [12,13,14]. Nordmark® Collagenase NB 6 GMP Grade is a natural mixture of *C. histolyticum*-based Collagenase C1 and C2, neutral protease, and clostripain. Nordmark® Collagenase NB 4G Proved Grade contains *C. histolyticum*-derived C1 and C2 collagenases blended with a balanced mix of neutral protease, clostripain, and trypsin-like activities [15,16,17]. In this study, we compare VitaCyte® GMP-grade Collagenase MA in combination with BP Protease to Nordmark® Collagenase NB 6 GMP Grade. Additionally, we compare non-GMP-grade Nordmark® NB 4G to GMP-grade Nordmark® NB 6 in an effort to identify a high-quality cost-effective alternative for hepatocyte isolation in non-clinical research applications. We hope the results of this study provide valuable information when selecting enzyme blends for hepatocyte isolations in clinical and non-clinical research applications.

3. Materials and Methods

3.1. Enzyme reconstitution & perfusion

All animal procedures were performed under the approved University of California, Irvine Institutional Animal Care and Use Committee (Protocol #: AUP-17-241). Our institution does not require ethical approval for reporting individual cases or case series. Additionally, there are no human subjects in this article and informed consent is not applicable. Hepatocytes were obtained from the livers of male Sprague Dawley rats weighing between 300-350g. Whole organ *in situ* digestions were performed with a GMP-grade VitaCyte® Collagenase MA (cat: 001-2030; lot: 122200903), and BP Protease (cat: 003-1000; lot: 211190815) blend, GMP-grade Nordmark® NB 6 (cat: N0002779; lot: 24130105), and non-GMP-grade Nordmark® NB 4G (cat: S1746501; lot: N18221). Lyophilized enzymes were reconstituted with Ca^{2+}-free Hanks' Balanced Salt solution® and used with the following enzyme concentrations: VitaCyte® Collagenase MA (2500 CDA U/mL) + BP Protease (550 NP U/ mL), Nordmark® NB 6 (0.125 PZ U/mL), and Nordmark® NB 4G (0.125 PZ U/ mL).

All livers were perfused using a modified version of Berry and Friend's two-step method[8]. Livers were perfused *in situ* via the hepatic portal vein with pre-warmed VitroPrep® Ca^{2+}-free Liver Perfusion Solution I (cat: CQ-PIB-1000) followed by perfusion with pre-warmed VitroPrep® Liver Perfusion Solution II (cat: CQ-PTB-1000) containing the previously aliquoted collagenases (Figure 1). The digested livers were removed, and cells were dispersed into a small volume of pre-warmed isolation media to isolate and purify hepatocytes. The isolation media contained Dulbecco's modified Eagle's medium (DMEM; cat: 11965-084; lot: 2192489) supplemented with 10% fetal bovine serum (FBS), 1% Insulin Transferrin Selenium (ITS-G), 1% Penicillin-Streptomycin, and 10 mM Dexamethasone.

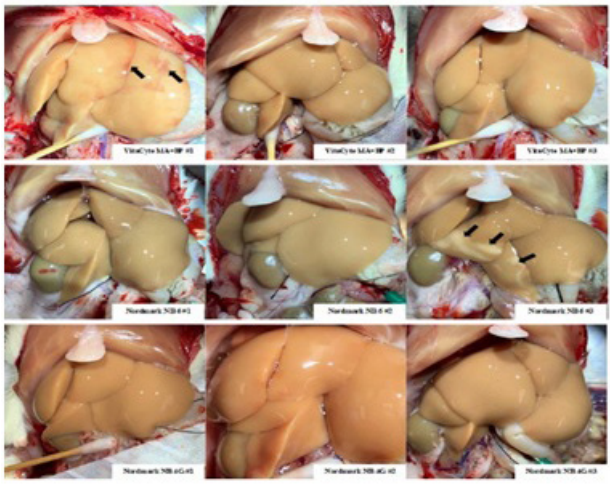

Figure 1: Photos taken during in-situ perfusion.

Photos were taken approximately 11 minutes after start of perfusion with the collagenase buffer solution. Each liver was perfused at 24 mL/min for approximately 15 minutes with Ca2+-free Liver Perfusion Solution I followed by perfusion with Liver Perfusion Solution II containing enzyme for 13 minutes. Arrows indicate areas of incomplete flushing of tissues.

3.2. Processing & purification

Each digested liver was strained through both a 250 and 80 µm nylon mesh, and fresh pre-warmed isolation media was added to the resulting suspension to a final volume of 300 mL. Trypan blue exclusion assay was used to assess pre-purification yield and viability. 70% viability was selected as the minimum allowable viability for inclusion in this study. Cell suspensions meeting this criterion were centrifuged at 100xg for 10 minutes, followed by supernatant aspiration and gentle re-suspension in warm media. Isotonic Percoll® gradient was selected for purification, followed by centrifugation at 100xg for 10 min. Supernatants were aspirated, and cell pellets were re-suspended in warm media followed by a trypan blue exclusion assay for yield and viability assessment post-Percoll®.

3.3. Cell culture

An aliquot of freshly-isolated hepatocytes was taken from the final purified cell suspension and diluted to a final concentration of $1x10^6$ cells/ mL with a pre-warmed William's-based (cat: A12176-01; lot: 2187335) hepatocyte plating media (10% FBS, 1% *GlutaMAX*™, 1% Penicillin-Streptomycin, 1% ITS-G, 10 mM Dexamethasone). Fresh cells were seeded in triplicate on 24-well type 1 collagen-coated plates, equating to a plating density of $2.11x10^5$ cells/ cm^2, and incubated at 37ºC in a 5% CO_2 environment. After four hours, the plating media was aspirated, and each well was rinsed 1x with 500 µL of a William's-based maintenance media (1% *GlutaMAX*™, 1% Penicillin-Streptomycin, 1% ITS-G, 10mM Dexamethasone). After rinsing, 500 µL of Gelterx®-supplemented (cat: A14132-02; lot: 2266435) maintenance media was added to each well, and the cells were incubated at 37ºC in 5% CO_2 for 72 hours. Media changes were performed at 24-hour intervals for three days (72 hours total), and the media was collected for albumin assay at the 72-hour time point. In addition to media collection, photographs of cultured cells were taken at 24-hour intervals to aid assessing cell polarization and morphology (Figure 2-4).

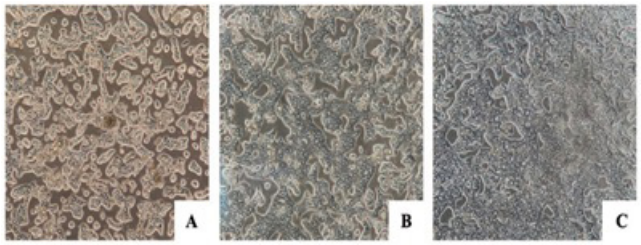

Figure 2: Mean pre- and post-purification viable yields of hepatocytes per gram liver based on digestion enzyme manufacturer type.

Two-sample Welch's t-tests indicated that the mean pre-purification viable yields of GMP-grade VitaCyte® and Nordmark® NB 6 were not significantly different (p=0.37, n=3), and the mean pre-purification viable yields of GMP-grade Nordmark® NB 6 and research-grade Nordmark® NB 4G were also not significantly different (p=0.86, n=3). Error bars are mean ± SEM. NS= not statistically significant.

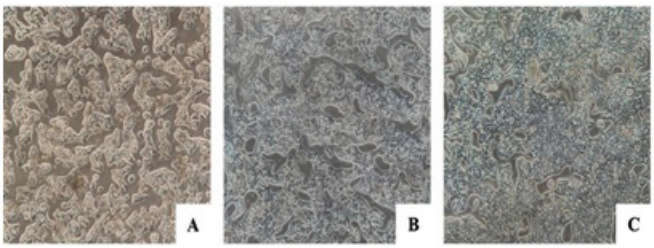

Figure 3: Mean pre- and post-purification viability of hepatocytes based on digestive enzyme manufacturer type.

A two-sample Welch's t-test indicated no significant difference in mean pre-purification hepatocyte viability between GMP-grade VitaCyte® and Nordmark® NB 6 groups (p=0.23, n=3), or mean pre-purification viability of GMP-grade Nordmark® NB 6 and research-grade NB 4G (p=0.10, n=3). Error bars are mean ± SEM. NS= not statistically significant.

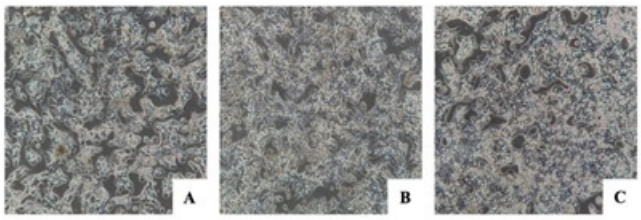

Figure 4: Select phase-contrast images of 24-hr hepatocyte cell cultures.

Cells isolated using **(A)**: GMP-grade VitaCyte® enzymes, **(B)**: GMP-grade Nordmark® NB 6, and **(C)**: research-grade Nordmark® NB 4G. Hepatocytes were plated at a density of $2.11x10^5$ cells/ cm^2. Images were taken at 10x objective.

3.4. Cryopreservation

Non-cultured hepatocytes were cryopreserved following a standard method. A volume containing 180 million viable freshly-isolated hepatocytes was taken from the final 100 mL purified cell suspension and centrifuged at 100 x g for 5 minutes. The resulting supernatant was aspirated, and the cell pellet was re-suspended in VitroPrep® NG5A CryoPreserv cryopreservation media (cat: CQ-NCP-100; lot: Z-19001) to a final concentration of 10^7 viable cells/ mL and aliquoted in 1.5 mL samples. The prepared samples were cooled to -90ºC in a controlled rate freezer (Thermo Scientific™), after which the samples were placed in vapor-phase LN2 at -190ºC for potential future analysis.

3.5. Cell Identification with Albumin ELISA

Freshly-cultured cells were positively identified as hepatocytes through ELISA assaying of culture media via Abcam® Rat Albumin ELISA Kit (cat: ab235642; lot: GR3363157-1). After 72-hours, culture media within each group was collected in triplicate and pooled. Fresh hepatocyte media, pooled media from each group and a protein standard were assayed in duplicate per manufacturer protocol. A 10-minute TMB incubation time was selected for this assay.

3.6. Statistical analysis

The data obtained from this study were analyzed using R software version 3.5.2, and all graphs were generated in Microsoft Excel®.

Mean pre-purification viabilities and yields of hepatocytes isolated from the three enzymes blends were compared using Welch's two-sample t-test, and p-values < 0.05 were considered significant. Mean liver mass, viability, and yield were calculated for each group using triplicate samples. Pre-purification data was tested for significance in order to assess the influence of each enzyme on hepatocyte viability and yield more directly. Mean rat albumin ELISA absorbance values in each group were calculated using duplicate samples per manufacture recommendation.

4. Results

4.1. Gross assessment of enzymatic liver tissue digestion

Photographs were taken of each liver approximately 11 minutes after the start of perfusion with collagenase-containing buffer II. A consistent color change in the liver lobes from reddish-brown to golden brown was observed in all isolations as tissue flushing and enzymatic tissue dissociation proceeded. However, there was a variation in color change which appeared as a striped pattern between the liver lobes of Nordmark® NB 6 isolation #3. Additionally, remnants of blood were visible within the liver lobes in VitaCyte® MA+BP isolation #1 (Figure 1). It is important to note that incomplete tissue flushing likely contributed to the slightly reduced final yields in these groups, although there was no statistically significant difference in mean viability or viable yields between groups overall.

Visually, enzymatic digestion of the liver under the Glisson's capsule appeared as tissue sloughing within the lobes as hepatocytes began to dissociate from one another. This was most evident in VitaCyte® MA+BP #2, Nordmark® NB 6 #1, Nordmark® NB 4G #2, and Nordmark® NB 4G #3 (Figure 1). Macroscopic tissue dissociation was evident in all groups approximately 10 minutes after the start of perfusion with buffer II.

4.2. Pre- and post- purification yields

The pre-purification viable yields of freshly-isolated hepatocytes were (56 ± 9.41), (68.2 ± 7.3), and (72.4 ± 12.9) million cells /g liver (mean ± SEM, n=3) for VitaCyte® MA+BP, Nordmark® NB 6, and Nordmark® NB 4G groups, respectively. The mean post-purification viable yields were (42.6 ± 12.5), (40.1 ± 5.7), and (45.3 ± 6.9) million cells /g liver (mean ± SEM, n=3) for VitaCyte® MA+BP, Nordmark® NB 6, and Nordmark® NB 4G groups, respectively. The results are summarized in (Table 1). A comparison of the mean pre-purification viable yields between VitaCyte® MA+BP and Nordmark® NB 6 showed no significant difference (p=0.37, n=3). Additionally, the mean pre-purification viable yields of non-GMP-grade NB 4G and GMP-grade NB 6 were not significantly different (p=0.80, n=3). Results are displayed in (Figure 5).

Table 1: Pre- and post-purification yields and viabilities of hepatocytes by collagenase blend manufacturer. All data represent mean values (n=3).

		Pre-purification				Post-purification			
Enzyme	**Mean liver mass (g)**	**Total yield (billion)**	**Viable yield (billion)**	**Viability (%)**	**Viable yield/ g liver (million)**	**Total yield (billion)**	**Viable yield (billion)**	**Viability (%)**	**Viable yield/ g liver (million)**
VitaCyte® (MA+BP)	13.9	0.918	0.77	83.3	56	0.618	0.577	93.1	42.6
Nordmark® NB 6	15.4	1.21	0.951	78.4	68.2	0.65	0.619	95.4	40.1
Nordmark® NB 4G	14.7	1.2	1.05	87	72.4	0.745	0.657	89	45.3

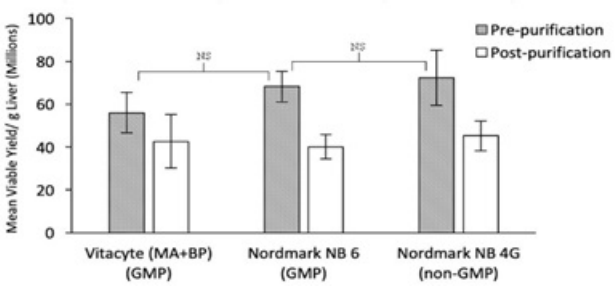

Figure 5: Select phase-contrast images of 48-hr hepatocyte cell cultures.

Cells isolated using **(A)**: GMP-grade VitaCyte® enzymes, **(B)**: GMP-grade Nordmark® NB 6, and **(C)**: research-grade Nordmark® NB 4G. Hepatocytes were plated at a density of $2.11x10^5$ cells/ cm^2. Images were taken at 10x objective.

4.3. Pre- and post-purification cell viability

The pre-purification viabilities of freshly-isolated hepatocytes in the VitaCyte®, Nordmark® NB 6, and Nordmark® NB 4G groups were (83.8% ± 3.0%), (78.4% ± 1.1%), and (87% ± 3.0%) (mean ± SEM, n=3). These increased to (93.1% ± 1.1%), (95.4% ± 1.4%), and (89% ± 3.0%) (mean ± SEM, n=3) in the VitaCyte®, Nordmark® NB 6, and Nordmark® NB 4G groups, respectively, after purification with 50% isotonic Percoll® (Table 1). Similar to mean viable yields, no significant differences were found between the mean pre-Percoll® viabilities of VitaCyte® MA+BP and Nordmark® NB 6 (p=0.23, n=3), and pre-Percoll® viabilities of Nordmark® NB 6 and Nordmark® NB 4G (p=0.10, n=3). Results are displayed in (Figure 6).

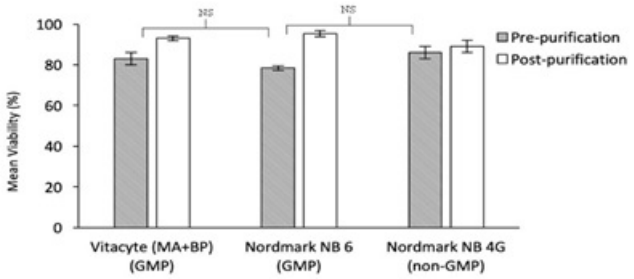

Figure 6: Select phase-contrast images of 72-hr hepatocyte cell cultures.

Cells isolated using **(A)**: GMP-grade VitaCyte® enzymes, **(B)**: GMP-grade Nordmark® NB 6, and **(C)**: research-grade Nordmark® NB 4G. Hepatocytes were plated at a density of 2.11x10^5 cells/ cm^2. Images were taken at 10x objective.

4.4. Morphological assessment of cultured cells

At 24-hours post-culture, VitaCyte® MA+BP, Nordmark® NB 6, and Nordmark® NB 4G-isolated rat hepatocytes were attached and formed a patched monolayer of cuboidal cells with defined edges and tight linear junctions [18,19].

The cell morphology was similar between the three enzyme blends with NB 4G-isolated hepatocytes appearing to have the highest confluency and the smallest unoccupied area between patches. Of the three groups, the VitaCyte® MA+BP isolated hepatocyte culture appeared to have the least amount of confluency. This pattern remained consistent in the 24-, 48-, and 72-hr post-culture images (Figure 2-4). In all groups, confluency increased with time during the 72-hour culture period, and cell polarization was evident in all the enzyme groups, with enhanced polarization of Nordmark® NB 4G hepatocytes compared to VitaCyte® MA+BP and Nordmark® NB 6 (Figure 2C-4C). Additionally, floating debris of non-viable hepatocytes was apparent 72-hr post culture in all the groups (Figure 4).

4.5. Hepatocyte identification with albumin ELISA

The albumin ELISA assay performed on hepatocyte culture media collected at 72 hours showed consistent albumin levels which ranged from 4.80 to 5.34 ng/mL in the three enzyme blend groups (n=2). Blank hepatocyte culture media was used as a control to exclude non-specific binding of residual serum albumin (Figure 7). ELISA testing of this media showed 8 to 9 times less albumin, 0.59 ng/mL, compared to media used to culture the isolated hepatocytes (n=2).

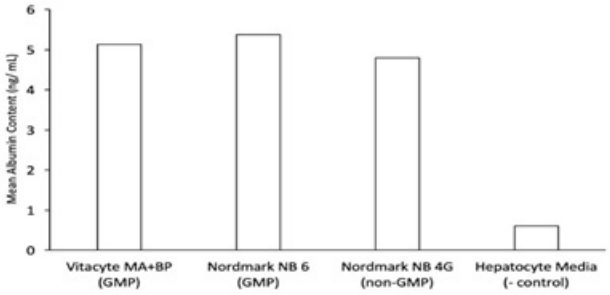

Figure 7: Results from 72-hr culture media albumin ELISA testing.

Data indicate the presence of albumin in culture media at 72 hours and confirm hepatocyte cell identification (n=2). Mean albumin content was consistent in media used in hepatocyte culture (5.13 ng/ mL), (5.34 ng/ mL), (4.80 ng/ mL), and (0.59 ng/ mL) in the Vitacyte®, Nordmark® NB 6, Nordmark® NG 4G groups, and negative control groups, respectively (n=2). A 10-minute TMB development solution incubation time was selected for this assay.

5. Discussion & Conclusion

Isolation of hepatocytes often employs bacterial-derived enzymes including collagenases and other proteases. However, non-GMP-grade collagenase preparations are non-standardized crude mixtures with variation in enzymatic activity, levels of endotoxin, and the presence of toxic proteolytic activities between lots. Therefore, the use of GMP-grade enzymes for hepatocyte isolations intended for clinical application is critical [20]. Utilization of GMP-grade enzymes to isolate hepatocytes for non-clinical purposes can be costly, however, necessitating investigation of cost-effective alternatives with comparable performance. Hepatocyte isolation outcomes between GMP-grade VitaCyte® Collagenase MA and PB Protease enzyme blend, GMP-grade Nordmark® Collagenase NB 6, and non-GMP-grade Nordmark® Collagenase NB 4G were compared. The results indicated that Nordmark® NB 6 performed similarly to VitaCyte® Collagenase MA and PB Protease enzyme blend. Additionally, non-GMP-grade Nordmark® NB 4G and GMP-grade Nordmark® NB 6 produced comparable isolation outcomes.

Although outcomes were similar between groups, the recommended enzymatic activities varied between manufacturers for rodent liver digestion. VitaCyte® recommends Collagenase MA be used at an activity of 2500 CDA U/mL (equivalent to 2.5 PZ U/mL) compared to the Nordmark® recommendation of 0.125 ± 0.025 PZ U/ mL for NB 4G and NB 6 collagenases [21,22]. The large difference in recommended activities is likely due to the mixture of natural enzymes, particularly clostripain, found in the Nordmark® blends. Liver tissue digestion and hepatocyte dissociation beneath Glisson's capsule was evident at the conclusion of enzymatic perfusion in all nine isolations. Visual differences in the appearance of the digested liver tissue between the isolations were potentially the result of variation in perfusion and cannulation techniques, both of which ultimately influence yield and viability of hepatocytes (Figure 1). Efforts were made to minimize variations between isolations by controlling for

temperature, digestion time, and enzyme concentration and flow rate. However, it was still not practical to completely control for perfusion and cannulation techniques; as a result, 70% pre-purification viability was selected as the minimum allowable viability for inclusion in this study to minimize the impact of perfusion technique on yield and viability of the isolated cells.

It was found that hepatocytes isolated with Nordmark® NB 6 had a greater mean pre-purification viable yield (68.2 ± 7.3) million cells /g liver compared to VitaCyte® MA+BP (56 ± 9.41) million cells /g liver (Table 1). The apparent lower average viable yield in the VitaCyte® MA+BP group was likely due to incomplete tissue flushing during step one of the perfusion of VitaCyte® MA+BP liver #1, although there was no significant difference in viable yield between these groups overall. Furthermore, a comparison between Nordmark® NB 4G and Nordmark® NB 6 showed a higher mean pre-purification viable yield in the NB 4G group (72.4 ± 12.9) vs. (68.2 ± 7.3) million cells /g liver. However, this difference was also not significant. This suggests that both the Nordmark® GMP and research-grade enzymes possess a comparable ability to liberate hepatocytes from liver tissue (Figure 5).

The data also revealed that hepatocytes isolated with Nordmark® NB 6 possessed a greater mean pre-purification viability compared to VitaCyte® MA+BP (83.8% ± 3.0%) vs. (78.4% ± 1.1%) respectively while hepatocytes isolated with Nordmark® NB 4G had the highest mean pre-purification viability overall (87% ± 3.0%). Although viability varied between groups, these differences were not statistically significant. Based on our findings, all three collagenases appear to be able to generate primary hepatocytes with comparably-high viability.

The Albumin ELISA assay on cell culture media from all enzyme groups showed greater mean albumin content compared to hepatocyte culture media alone. This finding helped to confirm the identity of the isolated and cultured cells in all enzyme groups as hepatocytes are the only cell type responsible for albumin production in the liver [23].

In summary, GMP-grade Nordmark® NB 6 and GMP-grade VitaCyte® MA+BP enzyme blends demonstrated comparable pre-purification viable yield, viability, and utility for isolation of primary rat hepatocytes. Therefore, GMP-grade Nordmark® NB 6 appears to be a potential alternative to GMP-grade VitaCyte® MA+BP enzyme blend for clinical applications of harvested hepatocytes. In addition, non-GMP-grade Nordmark® NB 4G and GMP-grade Nordmark® NB 6 produced primary hepatocytes with comparable pre-purification viable yield and viability. Thus, Collagenase NB 4G Proved Grade offers a reliable and affordable non-GMP grade collagenase alternative for non-clinical applications of isolated hepatocytes and R&D purposes.

6. Acknowledgement

The authors would like to acknowledge the support of the University of California, Irvine Department of Surgery, for providing their expertise and support throughout the writing of this manuscript.

7. Declaration of Conflicting Interests

Rafal Witek and Gabriel Peixoto were employees of Ambys Medicines, Inc. during the performance of this study. Johanna Mönch is employed by Nordmark Biochemicals who provided the enzymes tested in this study. All other authors declare no conflict of interest.

8. Funding Acknowledgement

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9. Ethical Approval

Our institution does not require ethical approval for reporting individual cases or case series.

10. Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the University of California Irvine, Institutional Animal Care and Use Committee, approved protocol number AUP-17-241.

11. Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

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