ORIGINAL ARTICLE

Quasi-experimental Clinical Trial on the Efficacy of In-house Collected Eggshell Membrane for the Management of Osteoarthritis.

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Abstract

Glucosamine products are widely used for the treatment of osteoarthritis (OA). Eggshell membrane (ESM) is a natural source of glucosamine, collagen, and hyaluronic acid, which are essential for cartilage health. ESM also possesses anti-inflammatory and immunomodulatory properties, that can help reduce pain and improve joint function in OA patients. This is a single-arm quasi-experimental clinical trial to determine the efficacy of in-house collected ESM in the management of OA. We prepared the ESM from eggshells, pasteurized it to eliminate bacterial contamination, and then packed it as a capsule containing 250 mg of ESM per capsule.
This study involved fifty-four participants with OA who met the eligibility criteria. They received two capsules of ESM daily for 30 days as a treatment. We used a questionnaire with a visual analogue scale (VAS) to measure their pain and stiffness levels at baseline and on the 10^{th}, 30^{th}, 60^{th}, and 90^{th} day after commencement of the treatment. The Friedman and post-hoc pairwise comparison Conover tests were used to test the significant reduction of the pain score. A *P*-value of 0.05 was taken as the significant limit. Of the fifty-four voluntary participants who enrolled in the study, three dropped out, resulting in a response rate of 94.4%. This study found that treatment with ESM significantly reduced pain scores for all 10 symptoms on day 30 compared to the initial baseline ($P < 0.01$). This improvement remained consistent on days 60 and 90 ($P>0.05$). Only one participant (1.9%) reported a 'warm sensation,' and no other adverse effects were reported. In-house collected ESM is a safe and effective alternative to glucosamine products for the management of OA. It can provide relief from pain and stiffness and improve joint function in OA patients.

Keywords: *egg shell membrane, inflammation, osteoarthritis, pain score.*

Introduction

Osteoarthritis (OA) is the 4th leading cause of disability among the major global health problems. It is more common in ageing populations, which are growing in Asia and other regions. As people live longer, OA is likely to become more prevalent. However, the mild smouldering inflammatory reactions are also implicated as the cause of OA. [1]

Globally, prevalent cases of OA increased by 113.25%, from 247.51 million in 1990 to 527.81 million in 2019. ASRs were 6,173.38 per 100,000 in 1990 and 6,348.25 per 100,000 in 2019, with an average annual increase of 0.12% [2].

There are different types of management for OA pain, such as non-pharmacological treatment, pharmacological treatment, and operative treatment. Non-pharmacological treatments such as weight reduction, non-weight bearing exercises, and lifestyle modifications are suitable for all patients with different stages of disease, from mild to very severe conditions. By following these treatment guidelines, the time needed to be operated on can be delayed. [3]

Analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) can provide symptomatic relief for osteoarthritis (OA) patients, but they do not alter the underlying disease process. NSAIDs also have potential adverse effects, such as gastrointestinal (GI) complications, which are more prevalent and severe in older adults. [3]

Hence, there is an unmet need for a novel therapeutic agent that can modify the disease course of OA without inducing harmful side effects, even with the long-term use.

Glucosamine sulphate, keratin sulphate, and chondroitin sulphate are currently widely used over-the-counter (OTC) drugs for joint health, assuming that they have the chondro-protective effect. They are expected to be in higher demand as arthritis and obesity become more prevalent globally. The US sales of these drugs were 810 million dollars in 2005. The study Glucosamine/chondroitin arthritis intervention trial (GAIT) by the National Institute of Health showed their benefits for arthritis. [4] In 2008, sixty one new products with glucosamine were launched worldwide. The global glucosamine market size was 29,087.3 tons in 2014. Japan's market was worth over 200 million dollars in 2014 [5]. The global glucosamine market size is projected to grow at a compound annual growth rate (CAGR) of 6.44% from 2023 to 2028 [6].

The efficacy of ESM products in OA management has been demonstrated by many previous clinical trials. These products reduced pain significantly and had a favourable safety profile, with rare occurrences of serious adverse reactions. [7,8,9,10,11,12,13]

Eggshell membrane (ESM) is a natural material that has potential benefits for the management of osteoarthritis. ESM is primarily composed of fibrous proteins such as collagen type I. [14] However, ESM also contains glycosaminoglycans, such as dermatan sulfate and chondroitin sulfate [15] and sulfated glycoproteins including hexosamines, such as glucosamine [16]. Other components identified in eggshell membranes are hyaluronic acid,[17] sialic acid,[18] desmosine and isodesmosine,[19] ovotransferrin,[20] lysyl oxidase,[21] lysozyme, [22] and β-N-acetylglucosaminidase.[23] Because of the presence of collagen, glucosamine, chondroitin, and hyaluronic acid together in the eggshell membrane as natural resources, the ESM becomes a potential material to replace the market of glucosamine for the treatment of joint and connective tissue pain.

ESM also has the ability to influence and regulate the immune system. [24] The whole ESM depressed inflammation by increased secretion of the anti-inflammatory cytokine IL-10 while the carbohydrate fraction of ESM reduced secretions of the pro-inflammatory cytokines IL-1β and IL-6. [24]

There are many methods to separate hard eggshell and soft eggshell membranes. It is very meticulous to separate manually one by one and it takes time. Some used non-chemical separators.

Joseph H. MacNeil, professor of food science at Pennsylvanian State University, developed a machine that used a delicate multi-bladed knife to scrape the membrane from the surface of shell fragments. [25] Some use a "dissolved air-flotation separation unit" to separate ESM, which was patented as "Eggshell membrane separation method" in the United States. [26]

As a chemical separator, Vladimir Vlad used acetic acid. The unseparated eggshells are placed in a fluid tank containing acetic acid, which causes cavitation to separate the eggshell membrane from the eggshell. [27] New method uses airflow in the eggshell membrane separation process. [28]

We improvised the method of collecting the ESM in our lab and prepared the ESM product in-house in UniKL-RCMP. The efficacy and safety of ESM collected by this in-house method have not been established. Therefore, we conducted a single-arm quasi-experiment study with the Before-and-After (BA) method to evaluate the efficacy and safety of locally sourced, in-house collected ESM on osteoarthritis patients.

Materials and methods

Study design

This study employs a single-arm quasi-experimental design; the before-and-after (BA) method. A cohort of voluntary participants was examined before the commencement and followed up four times during the 90-day period.

Ethical consideration

The project was conducted upon clearance with an approval letter (UniKLRCMP/MREC/2017/0027) from the UniKL-RCMP research ethics committee, [30], and the trial was conducted in accordance with Good Clinical Practice and the Declaration of Helsinki. Patients received information about the trial's purpose, procedures, risks, and benefits and gave their written informed consent before participating. Our trial adheres to the CONSORT standards for reporting randomized controlled trials.

Preparation of the research materials

Our research assistant acquired sturdy eggshells from local bakery shops and meticulously cleaned them by immersing them in saline solution. This process effectively eliminated lingering albumin and impurities, resulting in a thoroughly cleansed state.

Subsequently, the robust eggshells underwent a blending procedure, and the separation of the membrane was accomplished using the floatation method. The extracted eggshell membranes were carefully pasteurized and desiccated by subjecting them to a laboratory oven set at 60°C for a duration of 48 hours (figure 2). Finally, the dried eggshell membranes were transformed into fine powder and securely stored within sterile glass containers. After sterility testing, the powder was packed in capsules containing 250mg of ESM for consumption (figure 3).

Microbiological analyses for bacterial contamination

Sterility testing

For sterility testing of the dry powdered form of the ESM, 500 mg of the product was added to 10 ml of soya bean casein digest broth and incubated at 32.5^{o}C for 14 days. The cultures were observed several times during the incubation for the presence of any turbidity (WHO Document QAS/11.413 FINAL. Test for Sterility)

Microbiological testing for the presence of Salmonellae

About 25 g of the ESM product was added to 225 ml of buffered peptone water and incubated at 35° C for 16 to 20 hours. Following incubation, 0.1 ml of the pre-enrichment buffered peptone water was inoculated onto 10 ml of Rappaport-Vassiliadis (RV) Salmonella enrichment broth

and incubated at 41.5° C for 24 to 48 hours. Following incubation, the broth was subcultured by streaking onto XLD agar plates and incubated at 35° C for 18 to 24 hours (figure 4). Following incubation, the XLD plates were examined for bacterial growth.

Participants

The sample size was estimated using GPower version 3.1.9.7 (Faul et al., 2007) to determine the required sample size for dependent samples (Pre- and Post-treatment), Wilcoxon signed-rank test. The effect size was set to 0.5, based on Cohen's (1988) criteria for a medium effect. The significance level was set to $\alpha = 0.05$, and the power was set to 90%. The minimum sample size needed to detect a significant difference between the two related groups was N = 47 respondents. To account for an anticipated drop-out rate of 10%, the final sample size was increased by 10%, resulting in N = 52 participants.

Participants were enrolled through a non-probability sampling method, and participation was voluntary. Patient aged 25 years and above with osteoarthritis, diagnosed by a general practitioner, who are experiencing mild to moderate knee pain (Visual Analogue scale 3 to 8), excluding pregnant women, were recruited to the studies). Patients who were taking medication such as painkillers, non-steroidal anti-inflammatory drugs (NSAID), glucosamine sulphate, Artrodar, Piascledine, corticosteroid, and traditional medicines were either excluded or instructed to stop taking those drugs for one week before participating as volunteers. This wash-out period allowed for the effects of those drugs to dissipate. Other than that, those who had received recent injections of any medications into the joint cavity (e.g., intra-articular steroid injection for less than three months), were taking any disease-modifying drugs such as methotrexate within three months or had a known allergy to egg or egg products were excluded from this study.

Intervention

The intervention procedure was explained to patients, who then provided written informed consent to participate. The participants were given sixty capsules containing 250 mg of the in-house collected ESM to treat their joint pain. These ESM capsules were to be taken in two capsules (500mg) daily for 30 days after meals.

Twenty Paracetamol tablets, each containing 500mg, were provided as an emergency remedy in case the pain reached an intolerable level. Participants were instructed to record the date and quantity of tablets taken on such occasions. Additionally, participants could choose to discontinue their involvement in the study at any time.

Adverse event

The secondary objective of this study is to evaluate the tolerability and any adverse reactions associated with supplementation with ESM. The participants were required to report any discomfort or adverse events and would be assessed by a clinical investigator and followed up until the event was resolved.

Data collection instrument and method

We used a validated pain-score questionnaire, consisting of 10 questions (modified from the WOMAC score), related to pain levels during activities and stiffness (29). Patients rated their pain on a scale of 0-10, with 0 indicating no pain and 10 indicating the worst (Visual Analogue scale). Pain scores were assessed prior to supplementation as baseline scores (D0) and then followed up on the 10^{th} (D10) and 30^{th} day (D30) after the commencement of the supplementation with ESM. The supplementation was stopped after the 30^{th} day but followed up further on the 60^{th} (D60), and 90^{th} day (D90) to observe the persistence of the effect.

Data collection was carried out online because of the movement control order during the COVID-19 pandemic. We used WhatsApp to communicate with the participants and provide

them with the research information, the consent form, the instructions for using the Visual Analogue scale and the feedback form. The feedback forms were returned via WhatsApp or directly.

In exchange for subscribing to a three-month internet plan to use "WhatsApp" on their phones, participants received a token of appreciation upon successfully completing feedback responses.

Statistical analysis

Statistical analyses were performed using RStudio version 2023.06.1+524 (Mountain Hydrangea). Shapiro-Wilk test showed the outcome data was not normally distributed ($P<0.001$). As the data does not meet the assumptions of normal distribution, non-parametric statistical methods were used. The Friedman test, a non-parametric alternative to the one-way repeated measure ANOVA is used to test the difference in median scores of subjects during three or more time points. *P*-values were determined by the post-hoc pairwise comparison using the Conover test for dependent samples following a statistically significant difference as determined by the Friedman Test and representing treatment versus baseline. A *p*-value less than 0.05 is considered statistically significant.

At the start of the study, fifty-four eligible voluntary participants were enrolled. While the study was ongoing, three individuals withdrew from the research project; thus, the response rate was 94.4%. Of the three dropped-out participants, one participant ceased participation due to unmanageable pain and received treatment via intra-articular injection. Another participant underwent total knee replacement surgery, thus excluded from the study. One participant (1.9%) reported experiencing a “warm sensation” throughout their body which was presumed to be an adverse reaction to the ESM, resulting in the discontinuation of their participation. Other than that, no other signs and symptoms of adverse reaction were reported.

Among the fifty-one respondents who were followed through, slightly more than half were female. All participants were adults and older adults (Mean age=54.6, SD=14.2). The average age among male participants was younger than females, with mean ages of 49.0 years old (SD=15.5) and 58.6 years old (SD=12.1), respectively. Almost half of the respondents suffered bilateral knee pain, and slightly more than half suffered unilateral pain in the left or right knee (Refer to Table 1).

The baseline pain score ranged between 0 and 9. Participants may not have some symptoms; therefore, they rated it as 0. (Refer to Table 2).

The pain scores at baseline and D10 showed significant improvements for pain when walking on ground level (Q1), pain when using staircases (Q2), pain when putting on socks (Q8), and morning stiffness (Q10). The median scores for these questions were 3(IQR=2.0), 7(IQR=2.0), 1(IQR=3.0), and 3 (IQR=1.0), respectively at baseline and 3(IQR=1.5), 5(IQR=(2.0), 1(IQR=2.0), 3(IQR=1.5) respectively at D10 ($p < 0.05$). The pain scores at D30 showed further significant reductions for all questions (Q1-Q10) compared to the baseline ($P <0.01$) (Refer to Table 3 and Figure 1).

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On comparison of the pain scores between D30 and D60, and between D60 and D90, there were no significant changes in pain score in all questions, with the median pain score on D90 ranging between 0 and 2 ($P>0.05$), showing that there was no increase in pain within 2 months after the supplementation was stopped on D30 (Refer to Table 3 and Figure 1).

Discussion

This study showed evidence that the sole supplementation with the in-house prepared eggshell membrane 500mg daily reduced the pain scores of all activities and the stiffness of the participants with OA. These findings are consistent with the findings of an open-label clinical study by KJ Ruff et al. in 2009. They

reported that supplementation with Natural Egg Shell Membrane (NEM®, 500 mg), taken once daily, significantly reduced pain, both rapidly (seven days) and continuously (30 days). It was suggested that eggshell membrane (ESM) would be a possible new natural dietary supplement for the management of joint and connective tissue disorders. [7] Another study by Ulrich Danesh and colleagues in 2014 also reported the effectiveness of NEM® as a therapeutic option in reducing pain [8].

Supplementation of ESM capsules 500mg per day is proven safe. Other than one participant reported the "hot sensation", which was presumed to be an adverse effect, none reported any other adverse symptoms. Evidence from previous studies also showed that ESM is safe for consumption [8, 9]. Regarding the doses and toxicity, In 2012, KJ Ruff et al. published a research article, "Safety evaluation of a natural eggshell membrane-derived product", in "The Food and Chemical Toxicology Journal". They stated that NEM (brand name of ESM available in the market) has no cytotoxicity, no genotoxicity, and no oral toxicity at doses up to fifty times (50X) the clinically tested human equivalent dose. They recommended that NEM is safe as a supplement for human consumption at levels up to 500 mg/day [9].

According to this study, the pain level did not increase after discontinuing ESM supplementation. This suggests that the effect lasted for two months (as participants were followed up to 2 months after the treatment was stopped). This result agrees with a randomised control trial (RCT) that found taking 450mg of water-soluble eggshell membrane (WSEM) every day improved walking distance, pain, and joint stiffness from the fifth day of supplementation, and the improvement continued for three months [10].

The optimal dosage of supplementation for OA management is 500mg per day, which has been shown to be effective and safe in many previous clinical trials [9,13]. A recent randomized, double-blind, placebo-controlled study reported that a lower dose of 300mg of the mildly processed eggshell membrane (ESM) also improved pain and function in OA patients [12]. However, a higher dose of 500mg of ESM offers additional benefit. A dose-related study by Canovas et al. found that a higher dose of 500mg of ESM reduced knee pain and stiffness and increased quadriceps muscle strength [13].

ESM plays a dual role in OA management. It acts not only as a natural supplement for cartilage repair but also as an immunomodulator. ESM powder and its carbohydrate fraction modulates the immune system in monocytes and macrophage-like cells. They inhibited the inflammatory transcription factor NF-κB and down-regulated the expression of immune-regulating receptors such as toll-like receptor 4, ICAM-1, and CD44. However, they had different effects on cytokine secretion: ESM increased the anti-inflammatory cytokine IL-10, while the carbohydrate fraction decreased the pro-inflammatory cytokines IL-1β and IL-6 [24]. ESM also enhanced the immune response to vaccines and immunotherapy by stimulating antibody production and immune cell activation. This synergistic effect was called an immune-stimulatory, infection-protective effect [31]."

Conclusion

Our results indicate that self-collected eggshell membrane is as effective as other similar products in the market in alleviating the pain and stiffness of osteoarthritis. It is also safe to consume.

Limitation and constraint

Due to the restrictions imposed by the COVID-19 pandemic and the movement control order, we could not conduct face-to-face sessions with the participants. Instead, we resorted to online communication for interviews and used WhatsApp to collect feedback. The participants self-reported their feedback scores on the

research material's effect, which could potentially compromise the reliability of the scores since they were not assessed by professionals.
Another limitation pertain to the study's design. The lack of randomization and blinding may have introduced the Hawthorne effect.

Recommendation for further studies or research

A more valid measure of pain could be achieved by conducting face-to-face interviews with the participants at each of the five visits during the clinical trial, and by performing clinical assessment, including radiological analysis. Future research should employ randomized clinical trials with a larger number of participants and a two-month prescription of ESM to provide stronger, evidence-based outcomes.

Conflict of interest

This research was funded by a short-term research grant from the Universiti Kuala Lumpur, Royal College of Medicine Perak. There were no affiliations or relationships with any pharmaceutical companies. Therefore, we declare no conflict of interest.

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Table 1. The characteristics of the respondents

Characteristic		**Number**	**%**
Sex	Male	21	41.2
	Female	30	58.8
Mean age (SD)	All	54.6 (14.2)	
	Male	49.0 (15.5)	
	Female	58.6 (12.1)	
Laterality	Bilateral	22	43.1
	Left	16	31.4
	Right	13	13.0

Table 2. Baseline summary statistics of pain-score of each question.

No.	Question	Minimum	Maximum	Mean (SD)	Median (IQR)
Q1	Pain on walking at flat ground level?	3	8	4.04 (1.22)	3 (2.0)
Q2	Pain when using staircases?	3	8	5.92 (1.60)	7 (2.0)
Q3	Pain at rest?	0	7	1.45 (1.57)	1 (3.0)
Q4	Pain when sitting with legs bent for an extended period of time (i.e., travelling in a car)?	1	9	5.47 (1.97)	6 (3.0)
Q5	Pain on standing up from squatting position?	1	9	5.12 (2.48)	5 (4.0)
Q6	Pain when getting in and out of a car?	0	8	5.04 (2.37)	6 (4.0)
Q7	Pain on kneeling, or sit in the cross-leg position?	1	8	5.96 (2.14)	6 (4.0)
Q8	Pain when putting on socks?	0	9	3.88 (2.60)	3 (4.5)
Q9	Pain with light household chores (i.e., Sweeping, mopping, vacuuming, etc.)?	0	8	3.75 (2.00)	1 (1.0)
Q10	Early morning stiffness?	1	8	3.67 (1.73)	3 (1.0)

Table 3. Comparison of Baseline (D0) median pain-score and D10, D30, D60 and D90 post treatment with in-house collected ESM.

Questions	Baseline Median (IQR)	D10 Median (IQR)	***P*-value Baseline /D10**	D30 Median (IQR)	***P*-value Baseline/ D30**	D60 Median (IQR)	**P value D30/D 60**
Pain on walking at flat ground level?	3 (2.0)	3 (1.5)	**0.006***	2 (1.5)	**0.000***	2 (2.0)	0.115
Pain when using staircases?	7 (2.0)	5 (2.0)	**0.018***	4 (1.5)	**0.000***	3 (2.0)	0.109
Pain at rest?	1 (3.0)	1 (2.0)	0.353	0 (2.0)	**0.022***	0 (2.0)	0.395
Pain when sitting with legs bent for an extended period of time (i.e. travelling in a car)?	6 (3.0)	5 (2.0)	0.058	4 (3.0)	**0.000***	2 (2.0)	0.053
Pain on standing up from squatting position?	5 (4.0)	4 (3.0)	0.147	3 (3.0)	**0.000***	2 (2.0)	0.214
Pain when getting in and out of a car?	6 (4.0)	4 (2.5)	0.107	3 (2.0)	**0.000***	2(3.0)	0.080
Pain on kneeling, or sitting in the cross-leg position?	6 (4.0)	6 (3.0)	0.061	4 (3.5)	**0.000***	3 (4.0)	0.183
Pain when putting on socks?	3 (4.5)	3 (4.0)	**0.041***	1 (2.5)	**0.000***	1 (2.0)	0.469
Pain with light household chores (i.e., Sweeping, mopping, vacuuming, etc.)?	1 (1.0)	3 (3.5)	**0.060**	2 (2.0)	**0.000***	2 (2.0)	0.412
Early morning stiffness?	3 (1.0)	3 (1.5)	**0.009***	2 (1.0)	**0.000***	2 (1.0)	0.309

*P-values were determined by the post-hoc Conover test for dependent samples following a statistically significant difference as determined by the Friedman Test and represent treatment versus baseline. P<0.05 is taken as a significant difference.

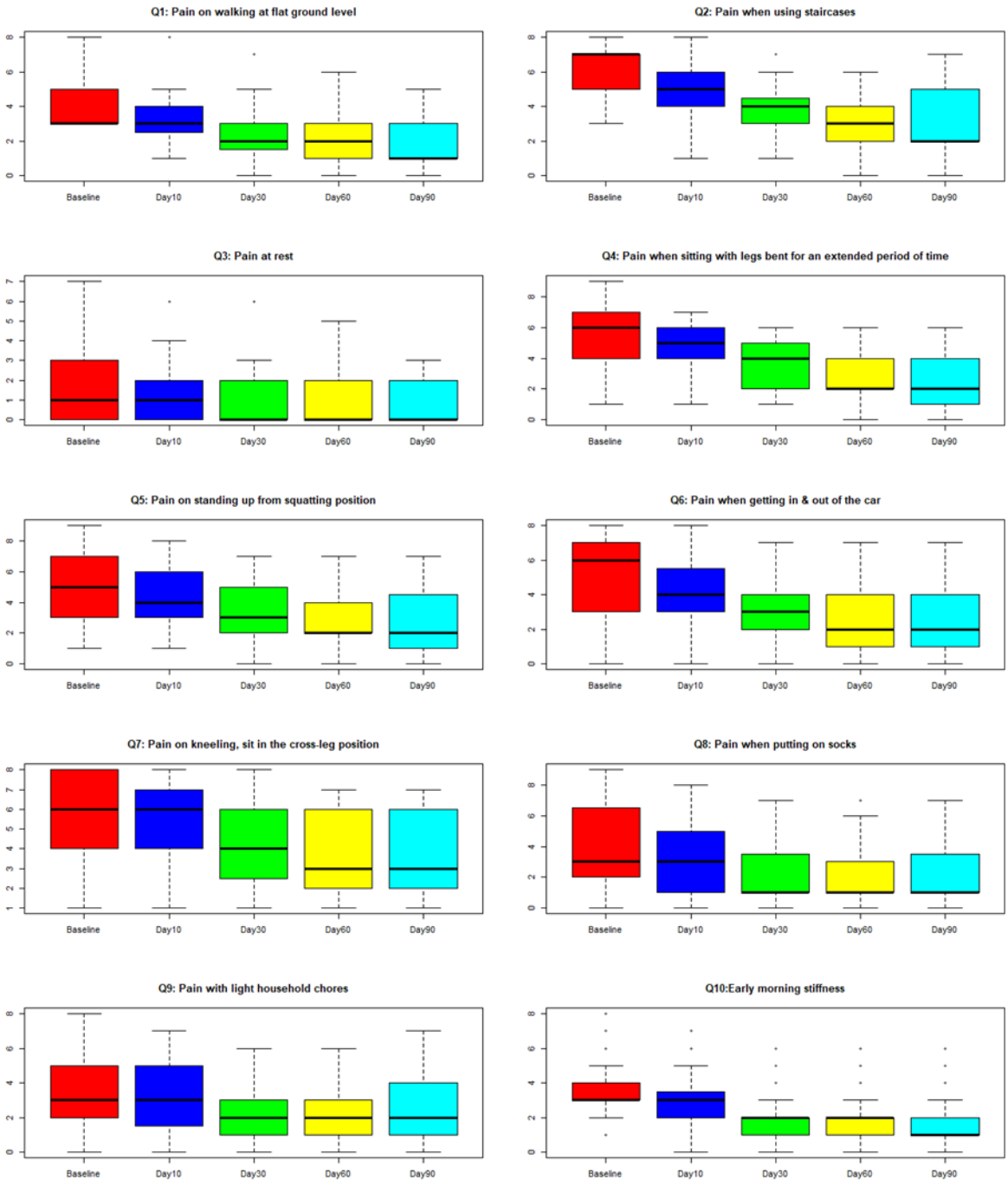

Figure 1. The box plot of pain score for each domain at baseline and followed-up

Figure 2. Making the in-house collected ESM dry inside the Oven

Figure 3. Encapsulation Manually by instructor

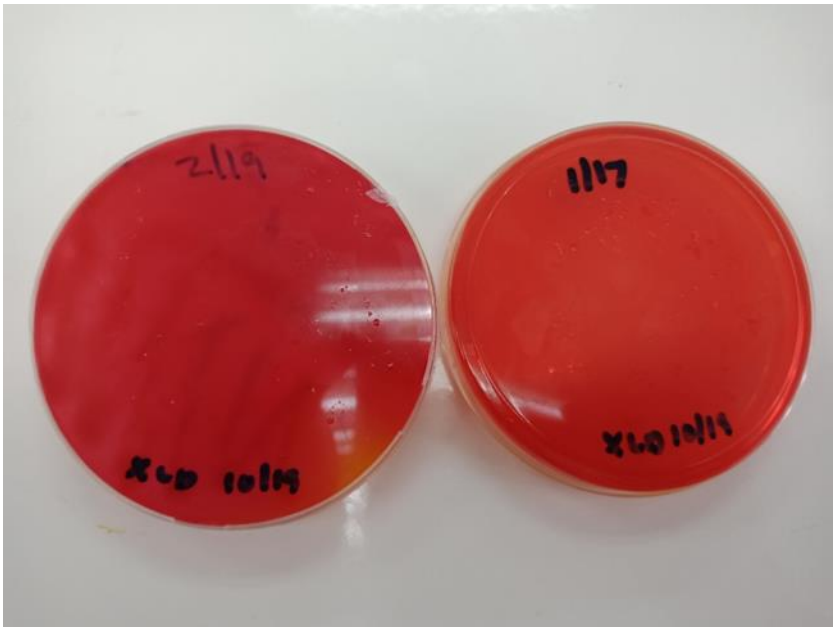

Figure 4. Prepared Culture media

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