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Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

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1 message

International Journal of Pharmaceutics <em@editorialmanager.com>

Wed, Feb 17, 2021 at 12:48 AM

Reply-To: International Journal of Pharmaceutics <ijp@elsevier.com>

To: Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Re: Thermosensitive and mucoadhesive in situ ocular gel for effective local delivery and antifungal activity of itraconazole nanocrystal in the treatment of fungal keratitis

Research Paper

Andi Dian Permana, Ph.D; Rifka Nurul Utami; Patricia Layadi; Achmad Himawan; Nana Juniarti; Qonita Kurnia Anjani; Emilia Utomo; Sandra Aulia Mardikasari; Andi Arjuna; Ryan F. Donnelly

Dear Dr. Permana,

Your submission entitled "Thermosensitive and mucoadhesive in situ ocular gel for effective local delivery and antifungal activity of itraconazole nanocrystal in the treatment of fungal keratitis" has been received by International Journal of Pharmaceutics

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Your Submission IJPHARM-D-21-00430

1 message

International Journal of Pharmaceutics <em@editorialmanager.com>
Reply-To: International Journal of Pharmaceutics <ijp@elsevier.com>
To: Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Sat, Mar 20, 2021 at 8:41 AM

Ms. Ref. No.: IJPHARM-D-21-00430

Title: Thermosensitive and mucoadhesive in situ ocular gel for effective local delivery and antifungal activity of itraconazole nanocrystal in the treatment of fungal keratitis
International Journal of Pharmaceutics

Dear Dr. Permana,

Comments on your paper have now been received and are attached to this message. Please consider all the points made and upon returning your paper detail your responses and the actions taken.

Please submit the revised manuscript by May 18, 2021. Upon receipt of your revised paper, we will inform you of the outcome as soon as possible. Papers not received within that that time period will be considered to be withdrawn, you are welcome to resubmit your paper as a new submission at a later date.

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Yours sincerely,

Fumiyoshi Yamashita
Editor-in-Chief
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Reviewers' comments:

Reviewer #1: The paper reports on the thermosensitive and mucoadhesive hydrogel loading itraconazole nanocrystals for potential therapy of fungal keratitis. I strongly suggest the author to improve the quality of the paper in the following items.

1. In abstract, "Itraconazole is a drug with low water solubility, which limits its absorption for ocular administration". Actually, the lipophilicity of drug significantly affect its absorption and bioavailability, rather than hydrophilicity.
2. In introduction section, the author should state why the F-127 thermosensitive hydrogel was used in this study? Is any favorable advantage over other types of thermosensitive hydrogel.
3. The procedures reported in Materials and Methods must be described in details. (in paragraph 2.3.1 author should indicate the Source of *Candida albicans*; in paragraph 2.3.2 the concentration of the DMSO is not specified; in paragraph 2.6.5 authors report "the amount of ITZ" without specifying if in volume or in weight, and so on).
4. It is recommended that the author should carefully check the format and writing specifications of the paper. (Some places are "ml" and some are "mL"; in paragraph 2.6.3 "each 0,5mL" written by the author may be changed into "each 0.5mL" and in paragraph 2.7.2 it may be changed into "1.5 mL of chloroform" and so on).
5. The amount of ITZ deposited in porcine eyes is described in Figure 6. It would be better expressed in the form of concentration, and it is amazing that the amount of drug can be reached at 8 mg both in infected cornea and normal cornea. So, author should be described the specific drug amount in situ gel you used in ex vivo ocularkinetic study and try to explain why there is no difference between infected and normal cornea.
6. The statistical analysis method should be stated in the manuscript
7. For the method of HPLC analysis, the related reference should be cited.

Reviewer #2: This work develop a nanocarrier formulation of itraconazole incorporated to a thermosensitive PF127 hydrogel for the treatment of ocular fungal infection. Overall, it is a well-written manuscript and recommended for publication after revision. Some specific comments are listed below

1. Section 3.2.1: "Increased solubilization of ITZ in NCs form would allow the drug to have a more extensive penetration into the fungal cell membrane" This sentence is a bit confusing. The increased solubilization is expected to increase the amount of free molecules rather than changing the mechanisms of action of the ITZ. I think the increased inhibition zone for the NC formulation is largely because the amount of free ITZ increased and comparable to the DMSO dissolve ITZ.
2. Section 3.2.2: "The excellent solubility of ITZ in DMSO helps enhance its penetration to reach the cell membrane." the function of DMSO is help solublizing ITZ rather than helping it to penetrate the cell membrane
3. It is recommended to study the dissolution rate of the NC formulation of ITZ to provide evident to what extent the NC formulation help ITZ to exert its antifungal activity.
4. "However, it is important to note that in all cases, the ratio between MFC and MIC was <4, which suggests the fungicidal activity of the drug (Tato et al., 2014)." is the sentence incomplete?
5. Figure 2a: it will be better to have a negative control of the DMSO to prove the DMSO has no impact on the antifungal activity of ITZ.
6. Section 3.3.1: "Interestingly, the effect of both types of Pluronic® F127 and F68 on the gelation temperature are the opposite of one another." F68 is commonly used to modulate the gelation temperature of F127. References should be included for this aspect.
7. Section 3.3.3: will pH adjustment of the thermogel solution be sufficient to ensure the final gel formulation is within the desirable pH if needed?
8. Section 3.3.4. it should be Figure 5
- 9: Section 3.4: " These three results prove the ability of the thermosensitive in situ ocular gel formulation to increase the contact time of the preparation with the ocular surface." these results didn't tell anything about the residence time of the thermogel on the ocular surface. instead, the results showing a extended period is required to release the incorporated ITZ in the thermogel. Discussion on the residence time of F127 thermogel on the ocular surface from the literature should be included and should compare with the drug release profile to anticipate will most of the ITZ be wasted without release.
- 10: what are the different between the data in Figure 7a and 7b? are they just different way of data interpretation? if yes, it is recommended to focus on one.

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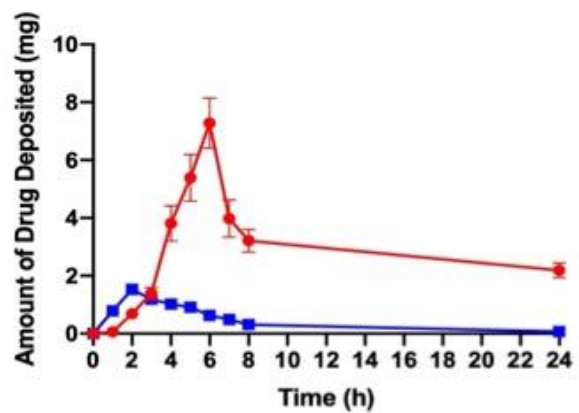
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International Journal of Pharmaceutics

Thermosensitive and mucoadhesive in situ ocular gel for effective local delivery and antifungal activity of itraconazole nanocrystal in the treatment of fungal keratitis --Manuscript Draft--

Manuscript Number:	IJPHARM-D-21-00430R1
Article Type:	Research Paper
Section/Category:	
Keywords:	itraconazole; nanocrystals; thermosensitive in situ ocular gel; fungal keratitis; mucoadhesive; antifungal
Corresponding Author:	Andi Dian Permana, Ph.D Hasanuddin University Makassar, INDONESIA
First Author:	Andi Dian Permana, Ph.D
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Abstract:	<p>Itraconazole is a lipophilic drug, which limits its absorption for ocular administration. This study focused on the incorporation of itraconazole into nanocrystalline carrier system with stabilizer Pluronic®F127 and was further formulated into thermosensitive in situ ocular gel. Itraconazole nanocrystals (ITZ-NCs) were fabricated using media milling method with ultra-small-scale device. The obtained nanocrystals were observed to have a better in vitro activity against <i>C. albicans</i> (CA) compared to free itraconazole suspension in water. Furthermore, the optimization of the thermosensitive ocular gel formula was carried out with a central composite design, using three types of polymers, namely Pluronic®F127, Pluronic®F68, and hydroxypropyl methylcellulose (HPMC). After being dispersed into the optimized thermosensitive gel base, ITZ-NCs did not alter in terms of physical characteristics. Ex vivo ocular kinetic studies on infected porcine eye models showed a better profile of the optimized formula of thermosensitive in situ ocular gel when compared to standard gel base. Importantly, the ex vivo antifungal activity of these preparations was also increased, with a 93% decrease in the CA population observed after 48 hours in infected porcine eye model. Altogether, this work has provided evidence of a novel approach in developing more advanced treatments for fungal keratitis.</p>

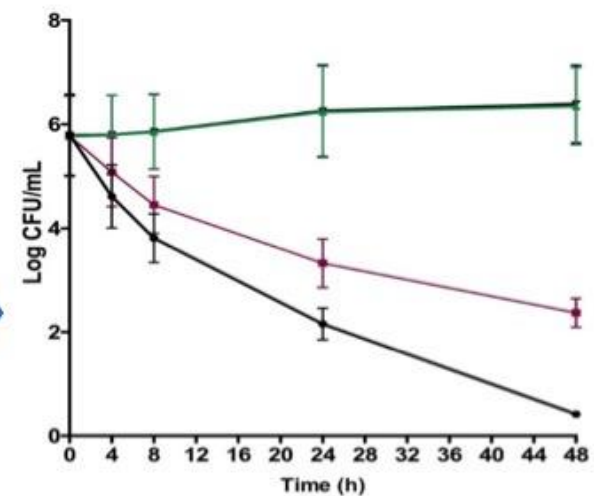
Graphical Abstract



Improved ocularkinetic profiles



Thermosensitive-mucoadhesive ocular gel



Improved *Ex Vivo* Antifungal Activity

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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The Editor

International Journal of Pharmaceutics

23rd March 2021

Dear Sir/Madam,

I wish you to consider our manuscript entitled: “**Thermosensitive and mucoadhesive *in situ* ocular gel for effective local delivery and antifungal activity of itraconazole nanocrystal in the treatment of fungal keratitis**” for publication in International Journal of Pharmaceutics.

We have addressed all of the comments raised by the Reviewer, substantially re-writing much of the manuscript, especially the Methods, Results and Discussion. We believe that the manuscript is now greatly improved. We have also made an effort to improve the scientific English of the manuscript.

I hope that you consider this manuscript worthy of publication in International Journal of Pharmaceutics and look forward to hearing from you in due course.

Yours sincerely,

Dr. Andi Dian Permana (on behalf of all authors)

Department of Pharmaceutics, Faculty of Pharmacy, Hasanuddin University, Makassar,
Indonesia

Email: andi.dian.permana@unhas.ac.id

Ms. Ref. No.: IJPHARM-D-21-00430

Title: Thermosensitive and mucoadhesive in situ ocular gel for effective local delivery and antifungal activity of itraconazole nanocrystal in the treatment of fungal keratitis
International Journal of Pharmaceutics

Response to Reviewers

We are very thankful to the expert reviewers for taking the time to kindly review our manuscript and provide helpful comments for improvement and clarification. We have made some changes to the manuscript as a result of these comments. We believe that the manuscript is now substantially improved. We have addressed each of the reviewers' comments in detail below.

Reviewers' comments:

Reviewer #1: The paper reports on the thermosensitive and mucoadhesive hydrogel loading itraconazole nanocrystals for potential therapy of fungal keratitis. I strongly suggest the author to improve the quality of the paper in the following items.

Response: We thank the reviewer for reviewing the manuscript in detail and providing valuable suggestions for the improvement of our manuscript.

1. In abstract, "Itraconazole is a drug with low water solubility, which limits its absorption for ocular administration". Actually, the lipophilicity of drug significantly affect its absorption and bioavailability, rather than hydrophilicity.

Response: We thank the reviewer for pointing this out. Indeed, the lipophilicity possesses more influence on absorption and bioavailability of drugs. Therefore, we have used the term "lipophilic" in the revised manuscript.

2. In introduction section, the author should state why the F-127 thermosensitive hydrogel was used in this study? Is any favorable advantage over other types of thermosensitive hydrogel.

Response:

We thank the reviewer for the suggestion. Following the reviewer suggestion, we have provided the detail of the use of Pluronic in the revised manuscript, as follows:

“The most frequently utilized thermosensitive polymers in pharmaceutical formulations, Poloxamers (known as Pluronics®), are amphiphilic synthetic copolymers comprising of polyoxypropylene oxide (PPO) block as hydrophobic part bounded by polyoxyethylene oxide (PEO) blocks as hydrophilic (Morsi et al., 2016). Because of their amphiphilic characteristic, Pluronics® can easily self-assemble to generate small micellar elements at low temperature. At higher temperature, they generate large micellar cross-linked network (Jiang et al., 2020). Importantly, in comparison with other thermosensitive polymers, such as polyester-based polymers, Pluronics® are reported to more stable from degradation in in vivo environment (Russo and Villa, 2019).”

References:

- Morsi, N., Ghorab, D., Refai, H., Teba, H., 2016. Ketorolac tromethamine loaded nanodispersion incorporated into thermosensitive in situ gel for prolonged ocular delivery. *International Journal of Pharmaceutics* 506, 57-67.
- Jiang, Q., Zhang, P., Li, J., 2020. Elucidation of Colloid Performances of Thermosensitive In Situ-Forming Ophthalmic Gel Formed by Poloxamer 407 for Loading Drugs. *Journal of Pharmaceutical Sciences* 109, 1703-1713.
- Russo, E., Villa, C., 2019. Poloxamer Hydrogels for Biomedical Applications. *Pharmaceutics* 11.

3. The procedures reported in Materials and Methods must be described in details. (in paragraph 2.3.1 author should indicate the Source of *Candida albicans*; in paragraph 2.3.2 the concentration of the DMSO is not specified; in paragraph 2.6.5 authors report "the amount of ITZ" without specifying if in volume or in weight, and so on).

Response:

We thank the reviewer for pointing this out. The details required have now been added into the revised manuscript.

- CA SC5314 (ATCC MYA-2876) purchased from (LGC Standards, Middlesex, UK) was maintained at 4°C

- For ITZ in DMSO, initially, ITZ were prepared in DMSO. Afterwards, the work solution was prepared by diluting the ITZ-DMSO solution in water. The final concentration of DMSO in the work solution was 1% v/v.
- The amount of ITZ in the thermosensitive *in situ* ocular gel was determined by dissolving a 1 mL of the *in situ* gel (equal to 10 mg of ITZ) in a 2 mL of acetone, then the mixture was sonicated for 1 hour in a bath sonicator.

4. It is recommended that the author should carefully check the format and writing specifications of the paper. (Some places are "ml" and some are "mL"; in paragraph 2.6.3 "each 0,5mL" written by the author may be changed into "each 0.5mL" and in paragraph 2.7.2 it may be changed into "1.5 mL of chloroform" and so on).

Response:

We thank the reviewer for pointing this out. We have re-read the manuscript thoroughly and changed the format mistakes accordingly.

5. The amount of ITZ deposited in porcine eyes is described in Figure 6. It would be better expressed in the form of concentration, and it is amazing that the amount of drug can be reached at 8 mg both in infected cornea and normal cornea. So, author should be described the specific drug amount in situ gel you used in ex vivo ocularkinetic study and try to explain why there is no difference between infected and normal cornea.

Response:

We thank the reviewer for the suggestion. Following the reviewer suggestion, we have provided the detail of the use of Pluronic in the revised manuscript, as follows:

“Then, 1 ml of the *in situ* gel (corresponding to 10 mg of ITZ) was placed on top of the donor compartment.”

“It has been reported that ITZ solubility was pH dependent, in which enhanced solubility was found at low pH (Yin et al., 2015). However, pH of tear fluid in mycotic keratitis was found to be around 7.15 (Norn, 1988), which is similar to normal tear fluid. Accordingly, the dissolution ability of ITZ in both cases might be similar, resulting in similar penetration profiles into cornea tissues.”

References:

Norn, M.S., 1988. Tear fluid pH in normals, contact lens wearers, and pathological cases. *Acta ophthalmologica* 66, 485-489.

Yin, X., Daintree, L.S., Ding, S., Ledger, D.M., Wang, B., Zhao, W., Qi, J., Wu, W., Han, J., 2015. Itraconazole solid dispersion prepared by a supercritical fluid technique: preparation, in vitro characterization, and bioavailability in beagle dogs. *Drug Des Devel Ther* 9, 2801-2810.

6. The statistical analysis method should be stated in the manuscript

Response:

We thank the reviewer for pointing this out. We have proved the statistical analysis in the method section.

7. For the method of HPLC analysis, the related reference should be cited.

Response:

We thank the reviewer for pointing this out. We have provided the related reference for the method of HPLC analysis.

Reviewer #2:

This work develop a nanocarrier formulation of itraconazole incorporated to a thermosensitive PF127 hydrogel for the treatment of ocular fungal infection. Overall, it is a well-written manuscript and recommended for publication after revision. Some specific comments are listed below

Response to Reviewer

We are very thankful to the Reviewer for taking the time to review this manuscript and for the expert review, providing helpful comments. We have made a number of key changes to the manuscript as a result of these comments. We believe that the manuscript is now substantially improved. We have addressed each one of the reviewers' comments in detail.

1. Section 3.2.1: "Increased solubilization of ITZ in NCs form would allow the drug to have a more extensive penetration into the fungal cell membrane" This sentence is a bit confusing. The increased solubilization is expected to increase the amount of free molecules rather than changing the mechanisms of action of the ITZ. I think the increased inhibition zone for the NC formulation is largely because the amount of free ITZ increased and comparable to the DMSO dissolve ITZ.

Response:

We thank the reviewer for pointing this out. Indeed, we agreed that the increased solubilization is not caused by the changing the mechanisms of action of the ITZ. Following reviewer comments, we have changed the discussion of this part, as follows:

"Increased solubilization of ITZ in NCs form would allow the drug to diffuse to the medium and to have a more extensive penetration into the fungal cell membrane (Koks et al., 2002; Niwa et al., 2014). Additionally, the increase of inhibition zone of NC formulation might be also caused by the higher amount of free ITZ in comparison with the free ITZ of ITZ coarse dispersion in water."

References:

Koks, C.H.W., Meenhorst, P.L., Bult, A., Beijnen, J.H., 2002. Itraconazole Solution: Summary of Pharmacokinetic Features and Review of Activity in The Treatment of Fluconazole-

Resistant Oral Candidosis in HIV-Infected Persons. *Pharmacological Research* 46, 195-201.

Niwa, T., Imagawa, Y., Yamazaki, H., 2014. Drug interactions between nine antifungal agents and drugs metabolized by human cytochromes P450. *Current drug metabolism* 15, 651-679.

2. Section 3.2.2: "The excellent solubility of ITZ in DMSO helps enhance its penetration to reach the cell membrane." the function of DMSO is help solublizing ITZ rather than helping it to penetrate the cell membrane

Response:

We thank the reviewer for pointing this out. Following reviewer comments, we have changed the discussion of this part, as follows:

"On the contrary, ITZ-DMSO showed very high antifungal activity as a result of high solubility of ITZ in DMSO."

3. It is recommended to study the dissolution rate of the NC formulation of ITZ to provide evident to what extent the NC formulation help ITZ to exert its antifungal activity.

Response:

We thank the reviewer for the suggestion. Following reviewer comments, we have provided the results of the *in vitro* dissolution of ITZ and ITZ-NCs in the revised manuscript.

4. "However, it is important to note that in all cases, the ratio between MFC and MIC was <4, which suggests the fungicidal activity of the drug (Tato et al., 2014)." is the sentence incomplete?

Response:

We thank the reviewer for pointing this out. Following reviewer comments, we have completed the sentence, as follows:

"However, it is important to note that in all cases, the ratio between MFC and MIC was <4, which suggests that ITZ exhibited fungicidal activity (Tato et al., 2014)."

Reference:

Tato, M., López, Y., Morosini, M.I., Moreno-Bofarull, A., Garcia-Alonso, F., Gargallo-Viola, D., Vila, J., Cantón, R., 2014. Characterization of variables that may influence ozenoxacin in susceptibility testing, including MIC and MBC values. *Diagnostic Microbiology and Infectious Disease* 78, 263-267.

5. Figure 2a: it will be better to have a negative control of the DMSO to prove the DMSO has no impact on the antifungal activity of ITZ.

Response:

We thank the reviewer for pointing this out. Following reviewer comments, we have now included the antimicrobial activity results of the solvents in the revised manuscript.

6. Section 3.3.1: "Interestingly, the effect of both types of Pluronic® F127 and F68 on the gelation temperature are the opposite of one another." F68 is commonly used to modulate the gelation temperature of F127. References should be included for this aspect.

Response:

We thank the reviewer for pointing this out. Following reviewer comments, we have now included the references related, as follows:

"Interestingly, the effect of both types of Pluronic® F127 and F68 on the gelation temperature are the opposite of one another. Khattab *et al.* also found that the addition of Pluronic® F68 into F127 was able to increase the gelation temperature of the formulation (Khattab *et al.*, 2019). Increase in the concentration of Pluronic® F127 lead to decrease in $T_{\text{sol-gel}}$ of resulting gel preparations. Conversely, the gelation temperature increased following the increasing concentration of Pluronic® F68. This phenomenon is considered to be due to the hydrophilic-hydrophobic structure difference between Pluronic® F127 and F68. The basic structure of Pluronic® is one PPO block as the center, flanked by two PEO blocks. The length of the blocks determines the type and physicochemical properties of the Pluronic® (Kozlov *et al.*, 2000)."

References:

Khattab, A., Marzok, S., Ibrahim, M., 2019. Development of optimized mucoadhesive thermosensitive pluronic based in situ gel for controlled delivery of Latanoprost:

Antiglaucoma efficacy and stability approaches. *Journal of Drug Delivery Science and Technology* 53, 101134.

Kozlov, M.Y., Melik-Nubarov, N.S., Batrakova, E.V., Kabanov, A.V., 2000. Relationship between Pluronic Block Copolymer Structure, Critical Micellization Concentration and Partitioning Coefficients of Low Molecular Mass Solutes. *Macromolecules* 33, 3305-3313.

7. Section 3.3.3: will pH adjustment of the thermogel solution be sufficient to ensure the final gel formulation is within the desirable pH if needed? (RIFKA)

Response:

We thank the reviewer for the comment. The pH for thermogel preparation in this study was predicted to be 7.38 following Design Expert analysis for the optimum formula. Measurements on the optimum thermogel formula showed pH value of 7.43 ± 1.21 . The statistical analysis also showed that out of three polymers, only Pluronic® F127 showed significant influence on the pH value of the preparation. On the other hand, it is possible if we are to carry out pH adjustment on the thermogel. Previous reports had successfully found that thermogel preparation using Pluronic® F127 could be prepared within desirable pH adjustment without reducing the ocular efficacy of the preparation. The aforementioned papers are (Abou el Ela and Khatib, 2014; Gugleva et al., 2020; Nagai et al., 2020):

References:

Abou el Ela, A.E.S.F., Khatib, M.M.E., 2014. Formulation and evaluation of new long acting metoprolol tartrate ophthalmic gels. *Saudi Pharmaceutical Journal* 22, 555-563.

Gugleva, V., Titeva, S., Ermenlieva, N., Tsibranska, S., Tcholakova, S., Rangelov, S., Momekova, D., 2020. Development and evaluation of doxycycline niosomal thermoresponsive in situ gel for ophthalmic delivery. *International Journal of Pharmaceutics* 591, 120010.

Nagai, N., Isaka, T., Deguchi, S., Minami, M., Yamaguchi, M., Otake, H., Okamoto, N., Nakazawa, Y., 2020. In Situ Gelling Systems Using Pluronic F127 Enhance Corneal Permeability of Indomethacin Nanocrystals. *Int J Mol Sci* 21, 7083.

8. Section 3.3.4. it should be Figure 5

Response:

Needful is done.

9: Section 3.4: " These three results prove the ability of the thermosensitive *in situ* ocular gel formulation to increase the contact time of the preparation with the ocular surface." these results didn't tell anything about the residence time of the thermogel on the ocular surface. instead, the results showing a extended period is required to release the incorporated ITZ in the thermogel. Discussion on the residence time of F127 thermogel on the ocular surface from the literature should be included and should compare with the drug release profile to anticipate will most of the ITZ be wasted without release. (RIFKA)

Response:

We thank the reviewer for pointing this out. Following reviewer comments, we have now provided more discussions in this part, as follows:

"These three results prove the ability of the thermosensitive *in situ* ocular gel formulation to enhance the ocularkinetic profile of ITZ. Indeed, this enhancement could be related to the ability of thermosensitive gel to increase residence time on ocular surface. Al-Khateb, *et al.* reported that Pluronic® F127-based thermosensitive gel was able to form *in situ* gel with even coverage and reside longer on the corneal surface following application (Al Khateb et al., 2016)."

Reference:

Al Khateb, K., Ozhmukhametova, E.K., Mussin, M.N., Seilkhanov, S.K., Rakhypbekov, T.K., Lau, W.M., Khutoryanskiy, V.V., 2016. *In situ* gelling systems based on Pluronic F127/Pluronic F68 formulations for ocular drug delivery. *International Journal of Pharmaceutics* 502, 70-79.

10: what are the different between the data in Figure 7a and 7b? are they just different way of data interpretation? if yes, it is recommended to focus on one.

Response:

We thank the reviewer for the suggestion. Indeed, Figure 7a and 7b are similar results with different way of data interpretation. We have now removed Figure 7b and focused on one figure.

Author Contribution Statement

Andi Dian Permana: Conceptualization, Review & Editing, Project Administration, Funding Acquisition, Validation, Supervision; **Rifka Nurul Utami:** Methodology, Writing - Original Draft; **Patricia Layadi:** Methodology, Investigation, Data Curation; **Achmad Himawan:** Conceptualization, Review & Editing, Supervision; **Nana Juniarti:** Methodology, Review & Editing, **Qonita Kurnia Anjani:** Data Curation, Review & Editing; **Emilia Utomo:** Data Curation, Review & Editing; **Sandra Aulia Mardikasari:** Data Curation, Review & Editing; **Andi Arjuna:** Data Curation, Review & Editing; **Ryan F. Donnelly:** Review & Editing.

1 **Thermosensitive and mucoadhesive *in situ* ocular gel for effective local delivery and**
2 **antifungal activity of itraconazole nanocrystal in the treatment of fungal keratitis**

3

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24 **Abstract**

25 Itraconazole is a lipophilic drug, which limits its absorption for ocular administration. This study
26 focused on the incorporation of itraconazole into nanocrystalline carrier system with stabilizer
27 Pluronic® F127 and was further formulated into thermosensitive *in situ* ocular gel. Itraconazole
28 nanocrystals (ITZ-NCs) were fabricated using media milling method with ultra-small-scale device.
29 The obtained nanocrystals were observed to have a better *in vitro* activity against *C. albicans* (CA)
30 compared to free itraconazole suspension in water. Furthermore, the optimization of the
31 thermosensitive ocular gel formula was carried out with a central composite design, using three
32 types of polymers, namely Pluronic® F127, Pluronic® F68, and hydroxypropyl methylcellulose
33 (HPMC). After being dispersed into the optimized thermosensitive gel base, ITZ-NCs did not alter
34 in terms of physical characteristics. *Ex vivo* ocularkinetic studies on infected porcine eye models
35 showed a better profile of the optimized formula of thermosensitive *in situ* ocular gel when
36 compared to standard gel base. Importantly, the *ex vivo* antifungal activity of these preparations
37 was also increased, with a 93% decrease in the CA population observed after 48 hours in infected
38 porcine eye model. Altogether, this work has provided evidence of a novel approach in developing
39 more advanced treatments for fungal keratitis.

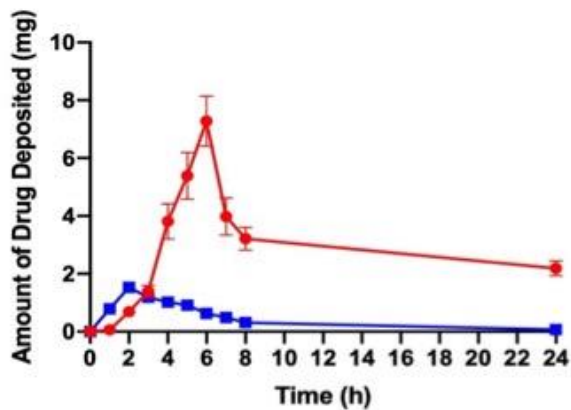
40 **Keywords:** itraconazole, nanocrystals, thermosensitive *in situ* ocular gel, fungal keratitis,
41 mucoadhesive, antifungal

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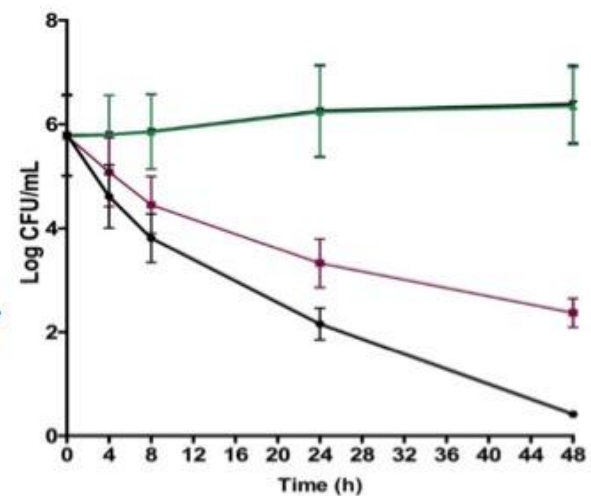
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Thermosensitive-mucoadhesive ocular gel



Improved ocularkinetic profiles



Improved *Ex Vivo* Antifungal Activity

45 1. Introduction

46 Fungal keratitis is an ocular inflammation, especially in the cornea, caused by fungal infection.
47 Fungal keratitis has become one of serious eye diseases because it can lead to blindness and total
48 loss of vision. Fungal keratitis may affect both male and female in either developing or developed
49 countries. In developed country such as the United States, the use of contact lenses becomes the
50 major cause of this condition. Conversely, in developing countries, ocular trauma caused by soil-
51 contaminated-object or plants has been reported as the main factor inducing this disease (Acharya
52 et al., 2017; Ansari et al., 2013). Most fungal keratitis is associated with the infection of *Fusarium*,
53 *Candida*, *Aspergillus*. This condition remains a challenge to clinicians due to delay in diagnosis
54 and difficulty in treatment which could aggravate the corneal ulcer (Anutarapongpan and O'Brien,
55 2014).

56 One of antifungal agents that has been widely used for the treatment of fungal keratitis is
57 itraconazole (ITZ). This drug shows a wide spectrum antifungal activity and has been used at the
58 dose of 200-400 mg/day (orally) and 1% (topically) to treat fungal keratitis (Miller and Alfonso,
59 2014). However, systemic therapy with ITZ is usually linked to some adverse effects, such as
60 gastrointestinal upset, headache, transient skin reaction and rare case of hepatitis (Gupta et al.,
61 2002; Lestner and Hope, 2013). Because of that, topical delivery of ITZ directly into the eye is
62 more preferable. Still, ITZ has very low solubility in water (1 ng/mL) and in acidic solution (4
63 µg/mL), which results in poor bioavailability and limits the number of ITZ topical preparations
64 available in the market (Permana et al., 2020b; Sahay et al., 2019).

65 To overcome those problems, nanocrystal (NC) approach can be utilized. NCs are nanoparticles
66 in the form of nanosized crystalline (200 to 500 nm) and composed of pure drug with stabilizers
67 on the surface. Nanoparticle-based drug delivery provides several advantages, such as improved
68 bioavailability, enhanced safety, higher drug loading, and most importantly increased solubility of
69 poorly soluble drug, including ITZ (Sharma et al., 2016; Zhou et al., 2017). Previously, we have
70 developed NC formulation of ITZ for improved efficacy in the *ex vivo* candidiasis skin infection
71 model (Permana et al., 2020b). Bearing this in mind, formulating the drug into NC may help
72 enhancing the efficacy following ocular administration.

73 Nevertheless, the anatomical and physiological structure of the eye may still hinder drug
74 absorption. Not only that, the eye also has a tear turnover mechanism which results in rapid
75 elimination of the drug (Macwan et al., 2016; Sharma et al., 2016). Previous studies had shown
76 two ways in increasing the bioavailability of drug in the eye; by using permeation enhancer, such
77 as cyclodextrin, ethylenediamine tetra acetic acid (EDTA), bile acid and its salts or by increasing
78 the viscosity of the preparations such as in the form of hydrogel or *in situ* gel (Kaur and Smitha,
79 2002). *In situ* gel is a liquid preparation that will transform into gel when exposed to physiological
80 conditions. The sol-gel transformation can be stimulated by body temperature, ionic strength of
81 biological fluids, and physiological pH. This type of preparation provides numerous benefits,
82 namely accurate dosing, control of drug release, and increased patient compliance due to less
83 administration frequency required (Soliman et al., 2019; Wu et al., 2019).

84 The most frequently utilized thermosensitive polymers in pharmaceutical formulations,
85 Poloxamers (known as Pluronic[®]), are amphiphilic synthetic copolymers comprising of
86 polyoxypropylene oxide (PPO) block as hydrophobic part bounded by polyoxyethylene oxide
87 (PEO) blocks as hydrophilic (Morsi et al., 2016). Because of their amphiphilic characteristic,
88 Pluronic[®] can easily self-assemble to generate small micellar elements at low temperature. At
89 higher temperature, they generate large micellar cross-linked network (Jiang et al.,
90 2020). Importantly, in comparison with other thermosensitive polymers, such as polyester-based
91 polymers, Pluronic[®] are reported to more stable from degradation in *in vivo* environment (Russo
92 and Villa, 2019).

93
94 This paper reports a formulation of ITZ-NC in a thermosensitive *in situ* ocular gel for increased
95 efficacy in the therapy of fungal keratitis. First of all, ITZ was made into a nanocrystal system to
96 increase its solubility. After that, we optimized the thermosensitive *in situ* gel formulation by
97 taking into account various parameters related to its physicochemical characteristics. Finally, the
98 optimized *in situ* gel formula was tested *ex vivo* to determine its ocularkinetic profile and anti-
99 fungal activity.

100

101

102 2. Materials and Methods

103 2.1. Materials

104 Itraconazole (ITZ) of analytical grade was obtained from Tokyo Chemical Industry (Tokyo, Japan)
105 with the purity of $\geq 98\%$. Pluronic[®] F127 (PF127) and HPMC K100 were obtained from Sigma–
106 Aldrich Pte Ltd, (Singapore, Singapore). Pluronic[®] F68 (PF68) was kindly gifted by BASF SE
107 (Jakarta, Indonesia). All other chemicals used in this study were of pharmaceutical grade.

108 2.2. Preparation and characterization of itraconazole nanocrystals

109 Itraconazole nanocrystals (ITZ-NCs) were prepared by a milling method using the ultra-small-
110 scale device according to method described in our previous study (Permana et al., 2020b). An
111 amount of 10 ml of 1% w/v Pluronic[®] F127 and 0.2 g ITZ were mixed in a 12 mL glass vial.
112 Afterwards, 8 g of zirconia beads were added into the vial. Then, the system was stirred with two
113 magnetic bars (12 x 6 mm) on basic magnetic stirrer (Thermo Scientific, Massachusetts, USA) for
114 6 hours at 1000 rpm. After that, in order to separate the zirconia beads and magnetic bars from the
115 formulations, the slurry was filtered (mesh 200 sieve). Finally, the NCs were washed three times
116 using distilled water at 14,000 rpm for 30 minutes.

117 Following preparation, the nanocrystals were characterized for the particle size and polydispersity
118 index (PDI) by dynamic light scattering (DLS) using a Zetasizer (Malvern Zeta Sizer, Malvern
119 Instruments, Malvern, UK). As a comparison, the characterization was also carried out after the
120 NCs were dispersed in the thermosensitive *in situ* ocular gel formulation. All of the measurements
121 were carried out at 25°C with equilibration time of 3 minutes.

122 To understand the dissolution rate of ITZ from NC formulation, the *in vitro* release study was
123 carried out using a dialysis method (Permana et al., 2019a; Permana et al., 2019b). Free-ITZ (10
124 mg) and ITZ-NCs which was equal to 10 mg of free-ITZ were dispersed into the dialysis membrane
125 12,000–14,000 MWCO (Spectra-Por[®], Spectrum Medical Industries, Los Angeles, CA, USA).
126 The dissolution study was conducted for 24 h at 100 rpm at 37°C in 100 mL of PBS (pH 7.4). To
127 ensure the sink condition, the dissolution media was 1% w/v of Tween 80. At predetermined time
128 intervals, 1 mL was collected, and 1 mL of fresh dissolution media was added to replace the media
129 collected. The amount of ITZ released from ITZ was quantified using HPLC.

130 **2.3. *In vitro* antifungal activity**

131 **2.3.1. Culture of *Candida albicans* (CA)**

132 CA SC5314 (ATCC MYA-2876) purchased from (LGC Standards, Middlesex, UK) was
133 maintained at 4°C. The fungal cultivation was carried out at 37°C and 100 rpm in Sabouraud
134 Dextrose Broth (SDB) for 24 hours. Fungal pellets were collected through centrifugation (3000
135 rpm for 30 min), then re-dispersed in fresh SDB. The optical density was set to equivalent to 1.5
136 $\times 10^7$ CFU/ml at 550 nm prior to the antifungal study.

137 **2.3.2. *In vitro* antifungal activity of ITZ-NCs**

138 The antifungal activity study of ITZ-NCs, ITZ in dimethyl sulfoxide (DMSO), and ITZ in water
139 against CA was carried out using the agar diffusion method. For ITZ in DMSO, initially, ITZ were
140 prepared in DMSO. Afterwards, the work solution was prepared by diluting the ITZ-DMSO
141 solution in water. The final concentration of DMSO in the work solution was 1% v/v. First of all,
142 the growth medium of Sabouraud Dextrose Agar (SDA) was prepared with the addition of
143 chloramphenicol (50 mg/mL) to prevent bacterial growth. Then, CA inoculum was spread all over
144 the agar on the petri dish. An amount equal to 2.5 µg/mL of free ITZ of the test materials was
145 placed on the paper disc. Afterward, the petri dishes were incubated for 48 hours at 32°C. The
146 inhibitory zone formed around the paper discs was measured using digital vernier calipers.

147 **2.3.3. Determination of minimum inhibitory concentration and minimum fungicidal 148 concentration**

149 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were
150 evaluated using microtiter broth dilution method. Three different preparations of ITZ were studied,
151 namely ITZ suspension in water, ITZ solution in DMSO, and ITZ-NCs. The experiment was carried
152 out as directed by the Clinical and Laboratory Standard Institute's Protocol. Briefly, fungal
153 suspension in SDB (100 µL) with concentration of 1.5×10^7 CFU/ml was placed in each bottom
154 of the microplates. Then, 100 µL of ITZ preparations in various concentration was added,
155 corresponding to 7.5×10^6 CFU/mL of fungal concentration. After that, the plates were incubated
156 at 37°C for 24 hours. MIC was determined based on the lowest concentration that inhibited fungal
157 growth following incubation. Whereas, the MFC was determined by cultivating 20 µl of dilutions

158 from MIC and concentration above the MIC wells into SDA plates and incubated at 37°C for
159 another 24 hours. The concentration in which 99.9% of fungal death was observed was determined
160 as the MFC.

161 **2.3.4. Time kill assay**

162 Time kill assay of ITZ and ITZ-NCs was performed three times according to previous method
163 (Permana et al., 2021; Permana et al., 2020a) with modifications. Dilutions of ITZ and ITC-NCs
164 with concentrations equivalent to MIC, 2x MIC, and 4x MIC were mixed with the CA fungal
165 dispersion to form 7.5×10^6 CFU/ml. Following incubation at 37°C for 24 hours, 20 µl of culture
166 media was collected at 0, 2, 4, 6, 8, 12, 18 and 24 hours. The culture was then cultivated into fresh
167 SDA plates and further incubated at 37°C for 24 hours. At last, the CFU on the SDA plates were
168 counted and the log CFU/ml was plotted against time-kill to produce the curve.

169 **2.4. Optimization of thermosensitive *in situ* ocular gel**

170 A central composite design (CCD) using Design Expert Software ver. 11 (State-Ease, Minneapolis,
171 MN, USA) was incorporated for the optimization of thermosensitive *in situ* ocular gel
172 formulations. The variables chosen in this study were the concentrations of the gel-forming
173 polymers, namely Pluronic® F127 (PF127), Pluronic® F68 (PF68), and HPMC (Table 1). The
174 concentrations were analyzed at three different level (low, medium, high). The response was
175 observed as the physicochemical characteristics of the resulting ocular gels, including sol-gel
176 temperature, mucoadhesion strength, pH, drug content, and viscosity at 25°C and 35°C. The
177 optimum formulation was determined as the *in situ gel* with sol-gel temperature at 35°C.

178 **Table 1.** The experimental design of ITZ-NCs thermosensitive *in situ* ocular gel formulation

Factors	Level		
	Low	Medium	High
Pluronic® F127 concentration (% w/v)	15.00	17.50	20.00
Pluronic® F68 concentration (% w/v)	0	3.75	7.50
HPMC K100 concentration (% w/v)	0.50	1.00	1.50

179

180

181

182 **2.5. Preparation of thermosensitive *in situ* ocular gel**

183 The thermosensitive *in situ* ocular gel of ITZ-NCs was prepared with varied polymer
184 concentrations based on the experimental design showed in Table 1. Firstly, specified amount of
185 HPMC was dissolved in water and slowly stirred until clear solution was obtained. After that,
186 Pluronic[®] F127 and F68 was added, and the mixture was placed in refrigerator overnight. The
187 mixture was stirred continuously until the entire components fully dissolved without any visible
188 lump. The amount of ITZ-NCs incorporated into the *in situ* gel was equal to 1% w/v of free ITZ.

189 **2.6. Characterization of thermosensitive *in situ* ocular gel formulations**

190 **2.6.1. Sol-gel transition temperature measurement ($T_{\text{sol-gel}}$)**

191 Sol-gel transition temperature was determined according to previous method (Khattab et al., 2019)
192 by placing a 2 mL of *in situ* gel in a closed-tube vial and heated in the water bath at 20°C. The
193 vials were rotated 90° every 1°C increment of the temperature until the bath temperature reached
194 65°C. $T_{\text{sol-gel}}$ was determined as the temperature at which the gel did not flow while the vial being
195 rotated. The test was performed in triplicate.

196 **2.6.2. Mucoadhesion strength**

197 Mucoadhesion strength was measured according to previously described physical balance method
198 (Morsi et al., 2016). Conjunctiva membrane extracted from porcine eye was used as a model
199 membrane. The membranes were fixed onto two separated glass vials with the mucosal side out.
200 One vial was connected upside down to a balance, while the other was put on a height-adjustable
201 pan. The gel was added onto the membrane of the first vial, and the height of the other vial was
202 adjusted so that the two mucosal surfaces came into contact and held for 2 minutes. After that,
203 weights were placed into the pan until the vials got separated. The minimum weight to separate
204 the vials was then calculated to obtain the mucoadhesion strength using the equation 1 below:

$$205 \quad \text{Mucoadhesion strength (dyne} \cdot \text{cm}^2) = \frac{m \cdot g}{A} \quad (1)$$

206 where, m is the weight required for detachment (g), A is the area of membrane exposed (cm²), g
207 is the gravity acceleration (980 cm/s²).

208 2.6.3. Viscosity

209 Rheological properties of *in situ* ocular gel of ITZ-NCs were observed using viscometer
210 (Brookfield DV III). Each 0.5 mL of the formula was used and put on the lower plate of the
211 apparatus, then, the viscometer was run with shear rate at 20 up to 500 s⁻¹. Rheology test was
212 performed using spindle no. 52 and at various temperatures (4 ± 0.1°C, 25 ± 0.1°C and 35 ± 0.1°C).

213 2.6.4. pH

214 pH of *in situ* ocular gel preparation is an important factor to ensure patient's comfort during
215 administration. The pH was measured using digital pH meter (Horiba Scientific, Kyoto, Japan)
216 and the test was run in triplicate.

217 2.6.5. Drug content

218 The amount of ITZ in the thermosensitive *in situ* ocular gel was determined by dissolving a 1 mL
219 of the *in situ* gel (equal to 10 mg of ITZ) in a 2 mL of acetone, then the mixture was sonicated for
220 1 hour in a bath sonicator. The mixture was then centrifuged for 15 minutes at 14,000 rpm.
221 Afterwards, the supernatant was collected to be analyzed using High Performance Liquid
222 Chromatography (HPLC). The percentage of drug loading was calculated using the following
223 equation:

$$224 \quad \text{Drug loading (\%)} = \frac{\text{Amount of encapsulated ITZ}}{\text{Total weight}} \times 100\% \quad (2)$$

225 2.7. Ocularkinetic study and antifungal activity in *ex vivo* model of infection on porcine eye

226 2.7.1. Preparation of *ex vivo* model of infection on porcine eye

227 Preparation of infected cornea was started by wounding porcine eye with a scalpel (3 slashes both
228 horizontally and vertically). Then on the corneoscleral button, a metal ring was placed to create a
229 watertight seal. Next, 10⁸ CA was added into the centre of the ring, or the organisms mentioned
230 above were injected intrastromally (using a 26-gauge needle; Becton Dickinson, Oxford, UK) with
231 the same number. Cornea that had been infecting were incubated at 37°C for 24 hours and were
232 then homogenized and diluted serially until the colony could be enumerated onto agar plates.

233 **2.7.2. *Ex vivo* ocularkinetic study**

234 *Ex vivo* ocularkinetic of thermosensitive *in situ* ocular gel of ITZ was examined using a Franz
235 diffusion cells with method described previously with slight modification (Permana et al., 2020b).
236 First, infected cornea membrane was connected using cyanoacrylate glue onto the donor
237 compartment of the cell. The cell was then arranged so that the donor compartment connected to
238 the acceptor part. Then, 1 ml of the *in situ* gel (corresponding to 10 mg of ITZ) was placed on top
239 of the donor compartment. The cell was put on magnetic stirrer and stirred at 600 rpm while the
240 temperature was maintained at $37 \pm 1^\circ\text{C}$. The cornea was removed at some predetermined time
241 points (0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24 and 48 hours) and rinsed with sterile water three times. A 1.5
242 mL of chloroform was added into an Eppendorf tube along with the cornea to extract the ITZ. The
243 sample was then vortexed for 15 minutes. Afterwards, the sample were centrifuged for 30 minutes
244 at 14,000 rpm and the supernatant was analyzed using HPLC. The ocularkinetic profiles were
245 evaluated using one-compartment open model of PKSolver (China Parmaceutical University,
246 Nanjing, China) (Zhang et al., 2010). The maximum drug concentration (C_{max}), the time of
247 maximum drug concentration (t_{max}), the drug concentration time curve from 0 to 72 hours (AUC),
248 the mean half-life ($t_{1/2}$) and the mean residence time (MRT) were all determined. As a comparison,
249 non-infected cornea was also used in this study and treated with the same procedure.

250 **2.7.3. Antifungal activity in *ex vivo* model of infection on porcine eye**

251 Antifungal activity was studied using a modified method (Permana et al., 2020b). Supernatant
252 collected from the ocularkinetic studies (24 and 48 hours) was used to evaluate the antimicrobial
253 activity of *in situ* ocular gel of ITZ-NCs against CA. A 20 μL of the supernatant was inoculated
254 into TSA plate then, was incubated for 24 hours at 37°C . As a comparison, a non-infected eye was
255 also used in this study and was treated same procedure as infected eye. At the end, the number of
256 viable CFU was counted.

257 **2.8. HPLC analysis**

258 Samples were analyzed using HPLC at ambient temperature using a Phenomenex[®] Luna C18
259 (ODS1) column with a particle size of 5 μm and size of 150 mm x 4,6 mm. Samples were injected
260 50 μL , then analyzed using mobile phase of acetonitrile and 25 mM ammonium acetate buffer

261 mixture with 65:35 v/v ratio of pH 5 solution. The mobile phase was run at a rate of 1 ml/min.
262 Next, the samples were detected at 270 nm using UV detector. Analytical method was validated
263 according to the International Committee on Harmonization (ICH) Guidelines 2005 (Permana et
264 al., 2020b).

265 **2.9. Statistical analysis**

266 All data were represented as means \pm standard deviation (SD) of the mean. SD of all results were
267 calculated using Microsoft[®] Excel[®] 2016 (Microsoft Corporation, Redmond, USA). The statistical
268 analysis was performed using GraphPad Prism[®] version 6 (GraphPad Software, San Diego,
269 California, USA). The p value < 0.05 indicated a significant difference.

270

271 **3. Results and Discussion**

272 **3.1. Characterization of ITZ-NCs**

273 The fabrication of ITZ-NCs in this present work was based on the optimization carried out in
274 previous study by Permana, *et al.* (Permana et al., 2020b). To ensure the reproducibility of the
275 utilized method and formulation, the ITZ-NCs were characterized for their particle size and PDI.
276 The characterization results show particle size of the obtained NCs of 378 ± 31 nm with the PDI of
277 0.35 ± 0.03 ($n=3$). Statistical analysis with previous report regarding this nanocrystal formulation
278 shows no significant difference ($p>0.05$), which suggests that the method and formulation were
279 reproducible.

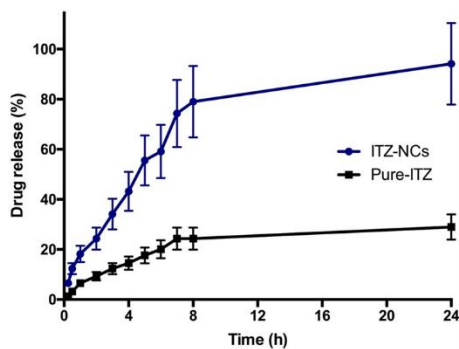
280 Figure 1 shows the *in vitro* release performance of ITZ from NCs formulation compared to free
281 ITZ. Based on this result, the dissolution rate of ITZ was significantly enhanced ($p < 0.05$) when
282 formulated into NC form. After 24 h, $94.17 \pm 16.32\%$ of ITZ was released from NC formulation.
283 On the other hand, the coarse ITZ dispersion was only release $28.98 \pm 5.04\%$ of ITZ after 24 h.
284 The enhancement of the dissolution rate in NC formulation was caused by the enlargement of
285 surface area of NC (Permana et al., 2020b).

286

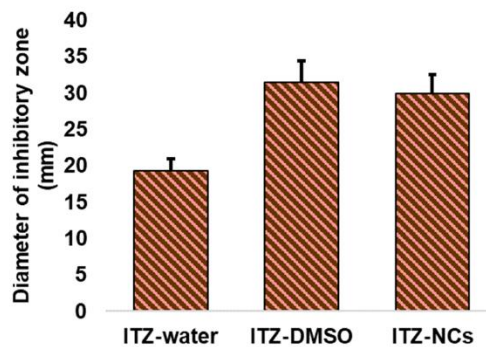
287 **3.2. *In vitro* antifungal activity**

288 **3.2.1. *In vitro* antifungal activity of ITZ-NCs**

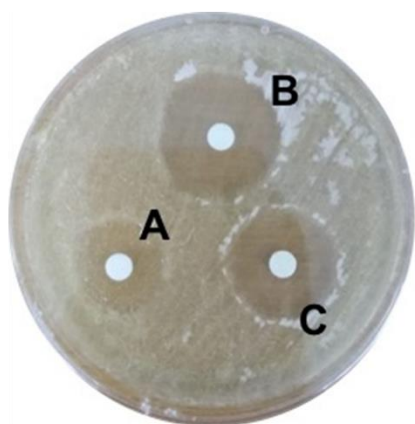
289 In this experiment, the antifungal activity of three different forms of ITZ against CA was studied,
290 namely ITZ suspension in water (ITZ-water), ITZ solution in DMSO (ITZ-DMSO), and ITZ-NCs.
291 The results of inhibitory zone of the different forms of ITZ are depicted in Figure 1. The free
292 suspension of ITZ in water had inhibitory zone diameter of 19.23 ± 1.76 mm. Remarkably,
293 formulation of ITZ into NCs was able to enhance the antifungal activity, increasing the inhibitory
294 zone diameter to 29.87 ± 2.65 mm. There were no inhibition zones found in water, 1% DMSO and
295 stabilizer solution of NC formulation (vehicle control) (Figure 1). Increased solubilization of ITZ
296 in NCs form would allow the drug to diffuse to the medium and to have a more extensive
297 penetration into the fungal cell membrane (Koks et al., 2002; Niwa et al., 2014). Additionally, the
298 increase of inhibition zone of NC formulation might be also caused by the higher amount of free
299 ITZ in comparison with the free ITZ of ITZ coarse dispersion in water. Accordingly, the inhibitory
300 zone diameter of ITZ-DMSO did not differ significantly from ITZ-NCs ($p > 0.05$) with the value
301 of 31.43 ± 2.98 mm. This also confirms that it is crucial for the drug to be present in soluble form
302 in order to exhibit its antifungal activity.



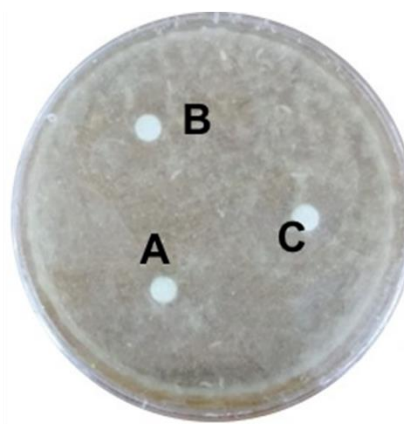
(a)



(b)



(c)



(d)

303 **Figure 1.** (a) *In vitro* dissolution of ITZ-NCs compared to pure ITZ ; (b) Diameter of inhibitory zone formed against
 304 CA of three different forms of ITZ (mean \pm SD, n = 3); (c) The inhibitory zone of (A) ITZ-water, (B) ITZ-DMSO,
 305 (C) ITZ-NCs; The inhibitory zone of controls
 306

307

308 **3.2.2. Determination of minimum inhibitory concentration and minimum fungicidal**
 309 **concentration**

310 The results for MIC and MFC are presented in Table 2. From the results, it is evident that the
 311 antifungal activity of ITZ-water is much lower than that of ITZ-DMSO and ITZ-NCs. The cause
 312 of this finding might be related to the hydrophobic nature of ITZ as explained previously. The
 313 antifungal mechanism of ITZ has been reported to be the inhibition of CYP450 enzyme on the
 314 fungal cell membrane. In order for the drug to exhibit its antifungal activity, it has to be able to
 315 penetrate to the cell membrane (Koks et al., 2002; Niwa et al., 2014). The penetration cannot occur
 316 if the drug presents as insoluble form, as per the case for ITZ suspension in water. On the contrary,

317 ITZ-DMSO showed very high antifungal activity as a result of high solubility of ITZ in DMSO.
318 Interestingly, similar results were observed for the ITZ-NCs. Indeed, this will uphold the previous
319 hypothesis that the incorporation of ITZ into NCs will increase the solubilization. Consequently,
320 this will result in enhanced antifungal activity of the drug. As for the MFC, all the obtained values
321 are higher compared to the respective MIC. However, it is important to note that in all cases, the
322 ratio between MFC and MIC was <4, which suggests that ITZ exhibited fungicidal activity (Tato
323 et al., 2014). Overall, these findings suggest that solubilization plays an important role in the
324 antifungal activity of ITZ. Based on the observations, the antifungal activity of ITZ-NCs was
325 comparable to that of free soluble ITZ in DMSO. This once again confirms that the formulation of
326 ITZ into NCs in order to increase the solubility can be advantageous for any therapeutic means
327 requiring this drug.

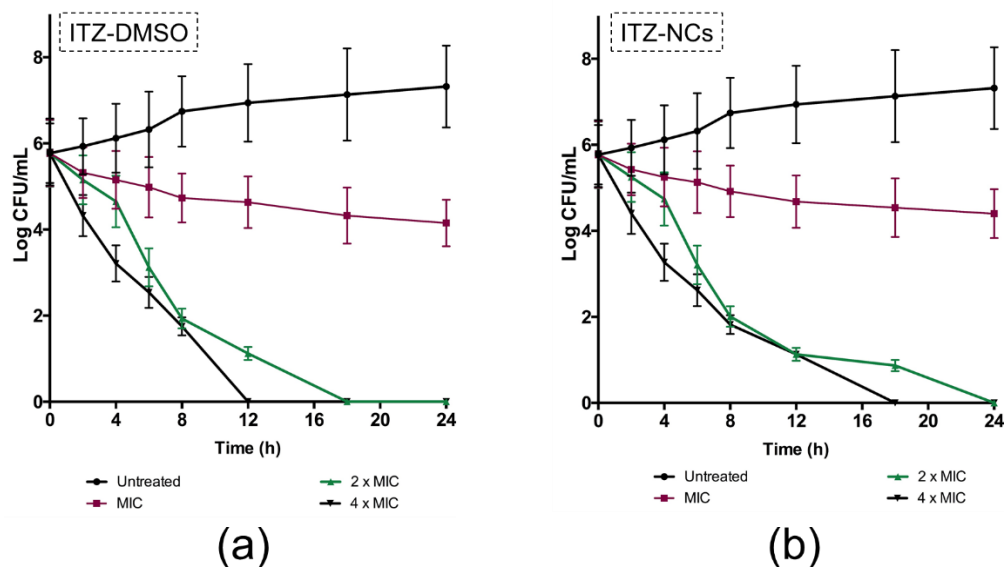
328 **Table 2.** MIC and MFC of three different forms of ITZ against CA (n = 3)

	MIC ($\mu\text{g/ml}$)	MFC ($\mu\text{g/ml}$)
ITZ-water	1280	2560
ITZ-DMSO	2.5	5
ITZ-NCs	2.5	5

329

330 3.2.3. Time kill assay

331 The time kill curves of ITZ-DMSO and ITZ-NCs against CA are presented in Figure 2. This
332 experiment was performed to observe the time required for an antifungal agent to eradicate fungal
333 growth completely. For the untreated group, the viable colony of CA reached 7.3 log CFU
334 following 24 hours of cultivation. It can also be seen from the result that at MIC, both ITZ solution
335 in DMSO and ITZ-NCs could not kill CA 99.9%. At the concentration 2x MIC, no viable CA was
336 observed after 18 hours and 24 hours for ITZ-DMSO and ITZ-NCs, respectively. Furthermore,
337 shorter time kill was required with concentration 4x MIC, 12 hours for ITZ-DMSO and 18 hours
338 for ITZ-NCs. From these observations, it can be stated that the fungicidal activity of ITZ is
339 concentration- and time-dependent. The results also emphasized the importance of ITZ solubility
340 in the fungal culture media to act as an antifungal agent.



341
342 **Figure 2.** Time kill assay of (a) ITZ-DMSO and (b) ITZ-NCs against CA (mean \pm SD, n = 3)

343

344 3.3. Optimization of thermosensitive *in situ* ocular gel

345 Characteristics of an excellent thermosensitive *in situ* ocular gel can be determined based on
 346 several parameters to ensure the efficacy of the preparation to deliver drug to the eye. One of the
 347 most important factors in the formulation is the gel-forming polymer used. In this study,
 348 combination of three polymers, namely Pluronic[®] F127, Pluronic[®] F68, and HPMC were utilized
 349 in the formulation of *in situ* ocular gel. Pluronic[®], also known as poloxamer, is a triblock
 350 copolymer consists of hydrophilic ethylene oxide (EO) and hydrophobic propylene oxide (PO).
 351 Pluronic-based polymer, especially the type F127, has long been used for the formulation of
 352 thermosensitive gel. However, Pluronic[®] F127 possesses a number of drawbacks, such as short
 353 residence time once applied onto the ocular mucosal layer (Soliman et al., 2019). Therefore, other
 354 polymers are often added into the formulation, including Pluronic[®] F68 and HPMC (Khattab et
 355 al., 2019; Kurniawansyah et al., 2020; Morsi et al., 2016), to improve the gel characteristics. Since
 356 each polymer has distinct chemical properties, it is expected that different concentrations will
 357 affect the gel formation. For the formulation optimization, a central composite design was used
 358 with varying the polymer concentration. The polymer concentrations were tested on three level
 359 each; Pluronic[®] F127 (15%, 17.5%, 20%), Pluronic[®] F68 (0%, 3.75%, 7.5%), and HPMC (0.5%,
 360 1%, 1.5%). The software suggested 15 formulas based on these compositions (Table 3). The

361 response recorded for the characteristics of resulting *in situ* gel, including gelation temperature
362 ($T_{\text{sol-gel}}$), mucoadhesion strength, viscosity, pH, and drug content.

363 **Table 3.** Variation of polymer concentration for the optimization of thermosensitive *in situ* ocular gel of ITZ-NCs

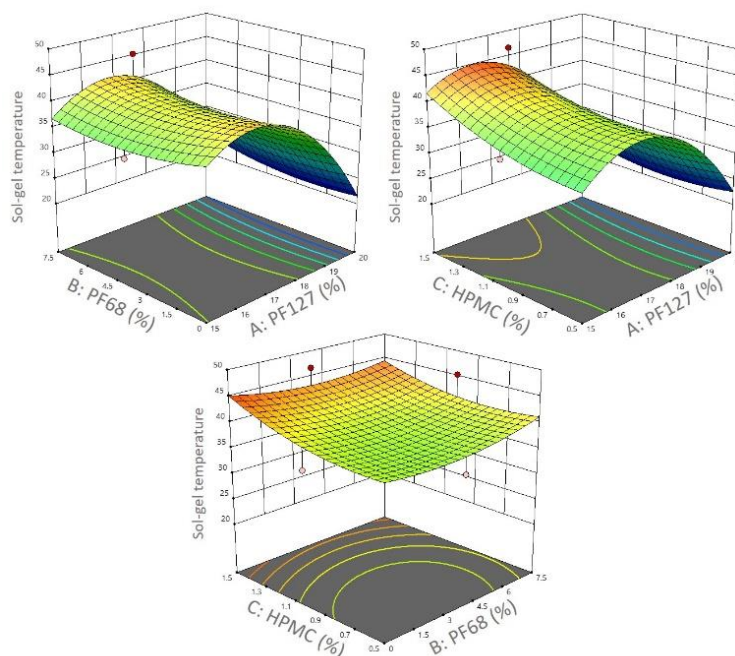
Formula	ITZ-NCs (equal to free ITZ)	Polymer Concentration (% w/v)		
		PF127	PF68	HPMC
F1	1	17.5	3.75	1
F2	1	20	3.75	1
F3	1	17.5	0	1
F4	1	20	0	0.5
F5	1	17.5	7.5	1
F6	1	20	7.5	1.5
F7	1	15	7.5	0.5
F8	1	20	0	1.5
F9	1	15	0	1.5
F10	1	15	0	0.5
F11	1	15	3.75	1
F12	1	17.5	3.75	0.5
F13	1	17.5	3.75	1.5
F14	1	20	7.5	0.5
F15	1	15	7.5	1.5

364

365 3.3.1. Effect of the polymer concentration on gelation temperature

366 Gelation temperature ($T_{\text{sol-gel}}$) is one of important parameters for the formulation of
367 thermosensitive gel. Formulation of thermosensitive *in situ* gel has to ensure that the preparation
368 remains liquid at room temperature to provide accurate dosing (Soliman et al., 2019). The average
369 temperature of mucosal layer in the eye is $\sim 34^{\circ}\text{C}$ (Tan et al., 2010), thus the acceptable range of
370 $T_{\text{sol-gel}}$ for thermosensitive *in situ* ocular gel is $30 - 35^{\circ}\text{C}$ (Khattab et al., 2019). $T_{\text{sol-gel}}$ values of
371 the 15 formulations in the optimization range between 22.3 to 46.6°C . The 3D graph representing
372 the effect of polymer concentration on $T_{\text{sol-gel}}$ is shown in Figure 3. Following the analysis,
373 Pluronic[®] F127 and F68 both showed significant effect on the gelation temperature ($p < 0.05$). On
374 the other hand, change in concentration of HPMC did not cause any significant alteration on the
375 $T_{\text{sol-gel}}$. Interestingly, the effect of both types of Pluronic[®] F127 and F68 on the gelation
376 temperature are the opposite of one another. Khattab *et al.* also found that the addition of Pluronic[®]
377 F68 into F127 was able to increase the gelation temperature of the formulation (Khattab et al.,

378 2019). Increase in the concentration of Pluronic® F127 lead to decrease in $T_{\text{sol-gel}}$ of resulting gel
379 preparations. Conversely, the gelation temperature increased following the increasing
380 concentration of Pluronic® F68. This phenomenon is considered to be due to the hydrophilic-
381 hydrophobic structure difference between Pluronic® F127 and F68. The basic structure of
382 Pluronic® is one PPO block as the center, flanked by two PEO blocks. The length of the blocks
383 determines the type and physicochemical properties of the Pluronic® (Kozlov et al., 2000). The
384 hydrophobic and hydrophilic portion ratios of Pluronic® F127 and F68 were 3:7 and 2:8,
385 respectively, causing Pluronic® F127 to be more hydrophobic (Zhang et al., 2017). The gelation
386 process of Pluronic involves dehydration of the hydrophobic PPO block, and hydration of the
387 hydrophilic PEO block resulting in the polymer to swell at certain concentration and temperature.
388 It is generally known that different PPO/PEO ratios will affect the gelation temperature of the
389 resulting gel. Hydrophobic PPO block will cause a decrease in the $T_{\text{sol-gel}}$, whereas hydrophilic
390 PEO will cause the opposite (Asasutjarit et al., 2011; Krtalić et al., 2018; M.A. Fathalla et al.,
391 2017).

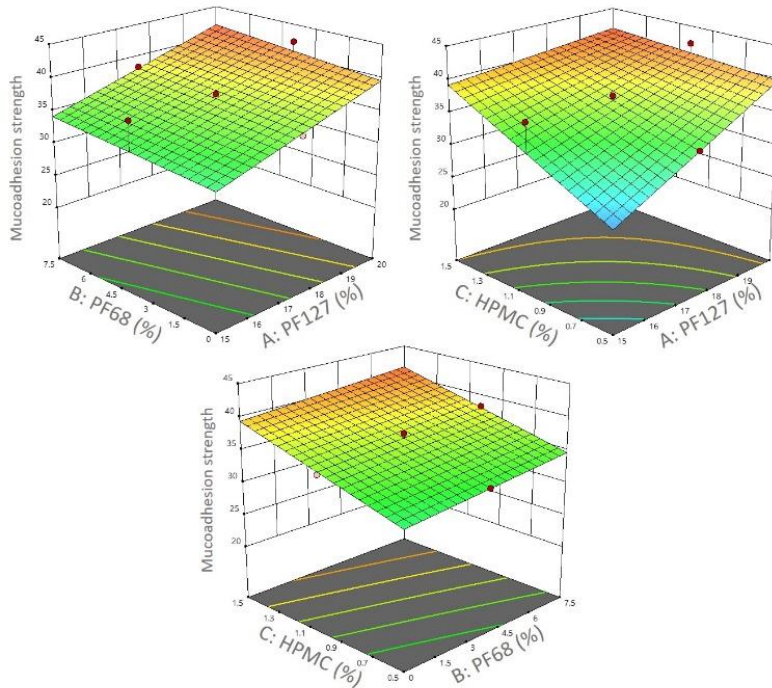


392
393 **Figure 3.** Representation response surface plots describing the effect of polymer concentration on the sol-gel
394 temperature of ITZ-NCs thermosensitive *in situ* ocular gel

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398 3.3.2. Effect of the polymer concentration on mucoadhesion strength

399 The contact time between the preparation and the ocular mucosal membrane also greatly
400 determines the bioavailability of the drug administered to the eye. Therefore, the characteristic of
401 a good ocular *in situ* gel is that it has sufficient mucoadhesion strength (Subrizi et al., 2019). The
402 effect of the polymer on the mucoadhesion strength of the thermosensitive *in situ* ocular gel in this
403 study is depicted on the 3D graphs in Figure 4. Of the three polymers that were varied in
404 concentration, only Pluronic® F127 and HPMC had a significant effect on the mucoadhesive
405 properties of the gel formulations ($p < 0.05$). Generally, polymers with excellent mucoadhesive
406 property are hydrophilic polymers, due to their ability to form hydrogen bond with mucin
407 (Andrews et al., 2009). Cellulose derivatives, including HPMC, have been widely reported to have
408 good mucoadhesive properties (Chowhan and Giri, 2020). The optimization results showed a
409 positive relationship between the increase in the HPMC concentration in the formulation and the
410 resulting mucoadhesion strength. Statistical analysis also showed a similar result with the increase
411 of Pluronic® F127 concentration, although not at the same extent as that of HPMC. Nevertheless,
412 various reports have shown that the use of Pluronic® F127 alone could not provide adequate
413 mucoadhesion (Kurniawansyah et al., 2020; Morsi et al., 2016; Üstündağ Okur et al., 2019). Thus,
414 the addition of other polymers such as HPMC is crucial for prolonging the residence time of the
415 dosage form on the ocular surface. Ocular preparations with desirable mucoadhesive properties
416 will help to avoid rapid drug elimination due to lacrimal drainage (Wu et al., 2019). This will
417 consequently lead to enhanced efficacy of preparations given through ocular route.



418
 419 **Figure 4.** Representation response surface plots describing the effect of polymer concentration on the mucoadhesion
 420 strength of ITZ-NCs thermosensitive *in situ* ocular gel

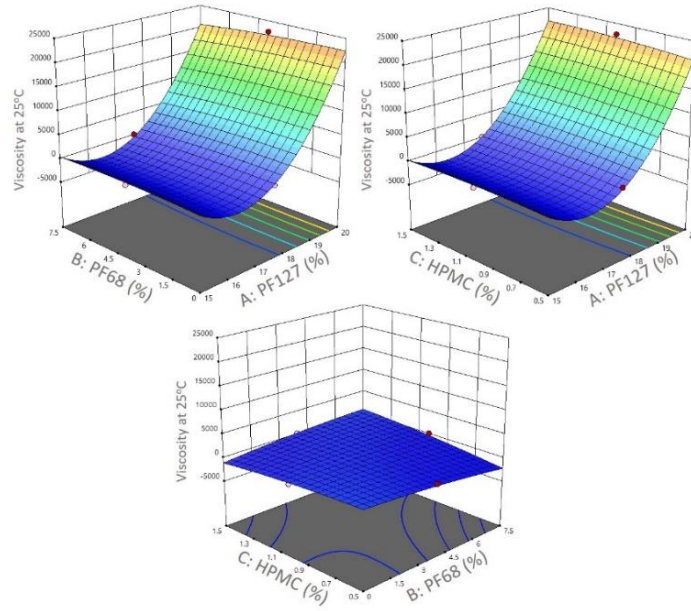
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422 3.3.3. Effect of the polymer concentration on pH and drug content

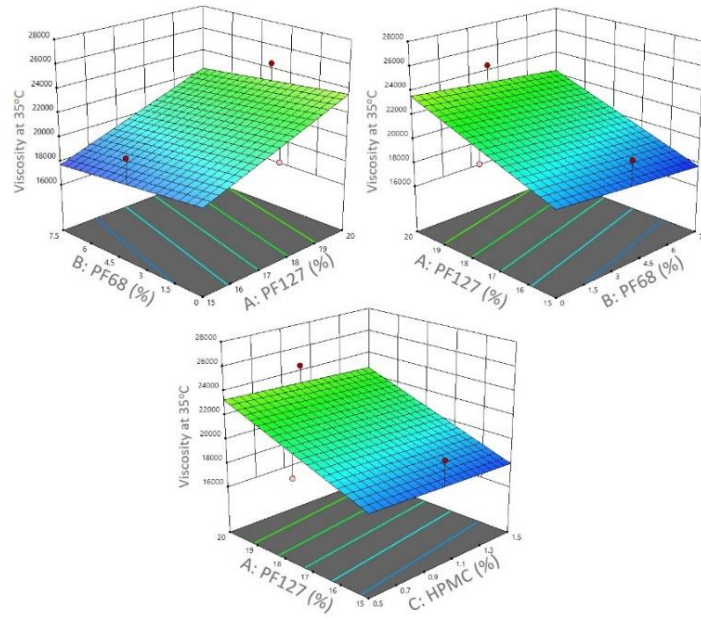
423 For ophthalmic preparations, pH is a factor that is closely related to patient compliance. Ideally,
 424 the pH of a preparation that is very well received by the eye is in the 6.5-8.5 range (Üstündağ Okur
 425 et al., 2019). pH that is too acidic or too alkaline will stimulate tears during application, thereby
 426 reducing therapeutic efficacy (Irimia et al., 2018). The results of this study indicate that of the 15
 427 optimized *in situ* ocular gel formulations, all the measured pH meets the requirement, with a value
 428 of 7.22-7.51. Statistical analysis showed that out of the three polymers, only Pluronic® F127 had
 429 a significant effect on the pH of the preparation. The measurement results showed that the pH will
 430 increase with the increase in the concentration of Pluronic® F127. On the other hand, the type and
 431 concentration of the gelling polymer used had no significant effect on the drug content. The value
 432 of the drug content recorded was 98.08-100.21%. Incorporation of ITZ into NCs is believed to
 433 increase its dispersibility, including in aqueous polymeric solutions. The drug content
 434 measurement data also shows that the *in situ* ocular gel preparations produced have uniform drug
 435 content, thus ensuring the dosage accuracy when given to the patients.

436 3.3.4. Effect of the polymer concentration on gel viscosity

437 One of the preferred attributes of *in situ* gel preparations is that they are in the form of a solution
438 at room temperature, but are able to form gel following contact with physiological conditions. The
439 solution form of the preparation during administration will help ensuring the dosage accuracy.
440 Therefore, it is important to compare the viscosity of the gel *in situ* preparation at the storage
441 temperature and the physiological temperature of the ocular membrane. Here, the viscosity of the
442 formulations was measured at 25° and 35°C. 3D graphs of the effect of polymer concentration on
443 the viscosity are presented in Figure 5. At 25°C, only the concentration of Pluronic® F127 has a
444 significant effect on viscosity, where the increase in polymer concentration also increases the
445 viscosity of the preparation. In fact, formulations with a high Pluronic® F127 concentration (20%
446 w/v) already formed gel at room temperature. Meanwhile, observations at 35°C indicated that the
447 concentration of Pluronic® F127 and F68, but not HPMC, had significant effect on the viscosity.
448 Interestingly, the effects caused by the two types of Pluronic were the opposite of each other. These
449 results are consistent with the measurement of gelation temperature, where a higher concentration
450 of Pluronic® F127 caused the gel to form at lower temperatures. The explanation for this
451 observation can be brought back to the nature of the Pluronic® F127 with higher hydrophobic chain
452 ratio than that of Pluronic® F68. The hydrophobic PPO chain will become dehydrated upon contact
453 with water. A higher extent of dehydration was found in Pluronic® F127 due to the longer PPO
454 chain, making this polymer more easily entangled, so that a gel structure can form at lower
455 temperatures (Morsi et al., 2016). Meanwhile, as previously described, Pluronic® F68 having a
456 lower hydrophobic/hydrophilic ratio is able to modify the gelation temperature of the *in situ* gel
457 formulation (Mahboobian et al., 2020; Morsi et al., 2016). Once again, this confirms that the
458 combination of Pluronic® F127 and F68 is very advantageous for *in situ* gel formulations to obtain
459 desirable viscosity, both at the time of storage and use of the preparations.



(a)



(b)

Figure 5. Representation response surface plots describing the effect of polymer concentration on the viscosity of ITZ-NCs thermosensitive *in situ* ocular gel at 25°C (a) and 35°C (b)

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469 3.3.5. Characterization of the optimized thermosensitive *in situ* ocular gel formulation

470 Following the optimization and data analysis process using the software, an optimized formula
471 was obtained. The *in situ* gel formulation was designed in such a way as to have gelation
472 temperature at 35°C, adequate mucoadhesion strength, suitable pH, high drug content and desirable
473 viscosity at 25° and 35°C. The optimum polymer concentrations as suggested by the software were
474 15.166 (% w/v), 5.629 (% w/v), and 1.105 (% w/v) for Pluronic® F127, Pluronic® F68, and HPMC,
475 respectively. As for the predictions and actual results of the characterization of this optimal
476 formula are presented in Table 4. Based on the test results, none of the optimal formula
477 characterizations deviates >15% from the predictions. Therefore, this optimal formula was used
478 for further investigation in this study.

479 **Table 4.** Predicted and actual value of the parameters following characterisation of the optimised formulation of
480 ITZ-NCs thermosensitive *in situ* ocular gel

Parameters	Predicted Value	Actual Value	Bias (%)
Sol-gel temperature (°C)	35.00	34.97 ± 0.39	-0.086
Mucoadhesion strength (dyne · cm ²)	43.657	43.91 ± 3.12	0.580
pH	7.38	7.43 ± 1.21	0.678
Drug content (%)	99.76	99.87 ± 1.02	0.110
Viscosity at 25°C (cps)	126.161	127.91 ± 11.21	1.386
Viscosity at 35°C (cps)	21584.431	22012.11 ± 1121.09	1.981

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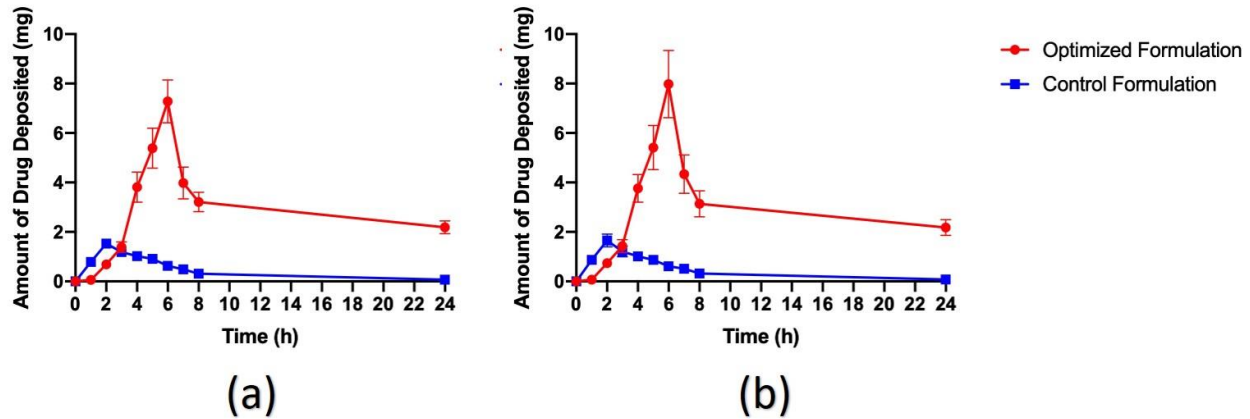
482 Along with that, the ITZ-NCs were characterized following dispersion in the optimized
483 thermosensitive ocular gel formulation. Encapsulation of ITZ into a nanocrystalline carrier aims
484 to increase its solubility. Therefore, it is important to ensure that the characteristics of the NCs do
485 not change after being dispersed into the polymeric gel base. After being dispersed to the optimized
486 *in situ* ocular gel base, the characterization results obtained were 389 ± 37 nm for particle size with
487 PDI 0.37 ± 0.03 (n = 3). From statistical analysis, it was found that there was no significant
488 difference from the initial fabrication of the nanocrystals ($p > 0.05$). From these results, it can be
489 concluded that the incorporation of NCs containing ITZ into an *in situ* ocular gel base consisting
490 of a mixture of Pluronic® F127, Pluronic® F68, and HPMC polymers did not alter the physical
491 characteristics of these NCs.

492 3.4. *Ex vivo* ocularkinetic studies

493 The main objective of ITZ formulation into thermosensitive *in situ* ocular gel preparation is to
494 increase the efficacy of therapy following administration. Hence, it is imperative to assess the
495 ocularkinetic properties of this preparation. The *ex vivo* ocularkinetic study of the thermosensitive
496 *in situ* ocular gel was carried out on porcine eye model infected with CA. Experiments were carried
497 out by comparing ITZ in NC formulation that was dispersed in thermosensitive gel and
498 conventional eye drop preparation as a negative control. In general, it was observed that the
499 thermosensitive gel formulation was able to increase ITZ absorption after ocular administration.
500 The peak concentration (C_{max}) of ITZ measured after administration of the thermosensitive gel and
501 control was 4.67 $\mu\text{g/ml}$ and 1.30 $\mu\text{g/ml}$, respectively. The time required to reach the peak
502 concentration (t_{max}) was 9.26 hours for the thermosensitive gel and 2.26 hours for control, with
503 mean residence time (MRT) of 18.62 and 4.53 hours, respectively. These three results prove the
504 ability of the thermosensitive *in situ* ocular gel formulation to enhance the ocularkinetic profile of
505 ITZ. Indeed, this enhancement could be related to the ability of thermosensitive gel to increase
506 residence time on ocular surface. Al-Khateb, *et al.* reported that Pluronic® F127-based
507 thermosensitive gel was able to form *in situ* gel with even coverage and reside longer on the corneal
508 surface following application (Al Khateb et al., 2016). The longer contact time consequently
509 increases the amount of drug that was deposited into the deeper eye tissues (Figure 6). This was
510 also evidenced by the significant difference ($p < 0.05$) of AUC of the drug measured in the
511 optimized and control, which were 85.94 $\mu\text{g/ml}\cdot\text{h}$ and 7.98 $\mu\text{g/ml}\cdot\text{h}$, respectively. Overall
512 measurement of ocularkinetic parameters was also performed in normal eyes where all values were
513 not significantly different from the infected eye. It has been reported that ITZ solubility was pH
514 dependent, in which enhanced solubility was found at low pH (Yin et al., 2015). However, pH of
515 tear fluid in mycotic keratitis was found to be around 7.15 (Norn, 1988), which is similar to normal
516 tear fluid. Accordingly, the dissolution ability of ITZ in both cases might be similar, resulting in
517 similar penetration profiles into cornea tissues.

518

519



520
521 **Figure 6.** Amount of ITZ deposited in (a) CA infected and (b) normal porcine eyes (mean \pm SD, n = 3)

522

523 3.5. Antifungal activity in *ex vivo* infection model on porcine eye

524 Finally, to prove the ability of this preparation as a therapeutic candidate for fungal keratitis,
525 antifungal activity study was carried out on CA cultures. The bioburden fungal observations
526 carried out after 48 hours showed that the incorporation of ITZ-NCs into the thermosensitive *in*
527 *situ* ocular gel possessed greater antifungal activity. The fungal burden observed in untreated
528 cultures increased from 5.8 log CFU to 6.4 log CFU. Based on the data shown in Figure 7,
529 administration of a thermosensitive *in situ* gel base (without ITZ) did not reduce the fungal
530 bioburden at all after 48 hours of incubation. In the administration of the control conventional eye
531 drop preparation, a decrease in the number of fungal populations was observed, but not statistically
532 significant, where the percentage decline in the population of CA was only 59% after 48 hours.
533 Meanwhile, application of thermosensitive *in situ* ocular gel containing ITZ-NCs showed
534 impressive results, where the percentage of the decline in the fungal population reached 63% after
535 24 hours. After 48 hours, a higher extent of reduction was observed of 93% (0.4 log CFU).

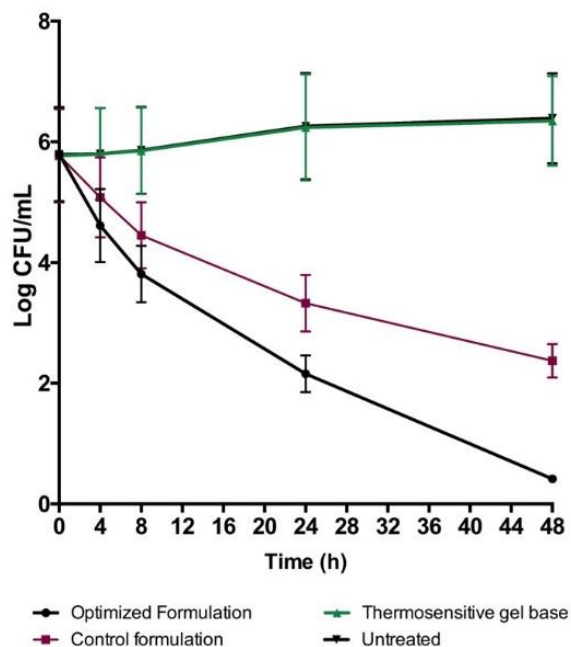


Figure 7. The results of *ex vivo* antifungal activity studies expressed in log CFU/ml and (means \pm SD, n = 3)

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539 From these findings, it is apparent that the optimized formulation of thermosensitive *in situ* ocular
540 gel of ITZ-NCs could enhance the delivery of the drug into the eye. The combination of
541 nanocrystal carrier and thermosensitive gel system provide better ocularkinetic profile of ITZ
542 which consequently increase the antifungal activity of the drug. This novel preparation evidently
543 possesses distinguished characteristic for the development of fungal keratitis treatment compared
544 to conventional eye drop. As an important future action, suitable animal model for *in vivo* study
545 should be carefully considered.

546 4. Conclusion

547 Altogether, the experimental results from this present work indicate that the formulation of ITZ
548 into NCs administered using a thermosensitive *in situ* ocular gel system shows superiority when
549 compared to conventional eye drop. Incorporation of ITZ into the NCs increased the solubility of
550 the drug, while further formulation into an optimized thermosensitive *in situ* ocular gel was found
551 to increase residence time in the eye. This of course proves that this preparation can be a good
552 candidate for the treatment of fungal keratitis. Further *in vivo* studies are highly recommended to
553 test the effectiveness of this therapy in more depth as an effort to develop more advanced
554 treatments for fungal keratitis.

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