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Effect of Different Levels of Hesperetin on the Quality of Rooster Semen

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ABSTRACT

The study examined the impact of varying concentrations of hesperetin on the cryopreservation of rooster sperm. Sperm were diluted with a Lake Extender containing (0, 15, 20, 25, and 30) µM hesperetin. After a 30-second freeze-thaw operation at 37 °C, membrane integrity, sperm motility characteristics, aberrant morphology, apoptotic status, and mitochondrial activity were assessed. The concentration of 25 µM hesperetin observed the highest values ($P < 0.01$) were (64.26 %, 30.44 %, 35.88 mm/s, 21.77 mm/s, 61.08 mm/s, 35.66%, 61.14%, 16.23 Hz, 61.71%, 61.71%, and 61.33%) for (Total motility, progressive motility, average velocity path, curved velocity, straight-line velocity, linearity, straightness, beat cross frequency, membrane integrity, mitochondrial activity, and viability, respectively). In contrast, it recorded the lowest values (3.94mm, 13.67%, 13.30%, and 25.37%) for lateral head displacement amplitude, total abnormality, apoptotic sperm, and dead sperm, respectively. It may be concluded that adding 25 µM hesperetin is an effective method for preserving the quality of cryopreserved rooster sperm.

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Keywords: Hesperetin, Sperm, Rooster, Spermatozoa, Cryopreservation.

1. Introduction

The poultry industry's success primarily depends on the reproductive health of the birds[1]. Cryopreservation is an efficient and well-known technique that conserves the variation in bird populations' genetic makeup, particularly those of endangered and essential species. This approach is not frequently used in the poultry industry; however, sperm quality drastically diminishes in preserved samples[2].

Sperm with average motility and good viability are essential for success in chicken production. Only motile sperm can traverse the vaginal canal. Many variables affect sperm motility, including environment, nutrition, and physiology[3, 4]. Oxidative stress also affects reproductive performance. The high fatty acids in the sperm plasma membrane are sensitive to oxidative stress and have a negative link with sperm motility and viability[5].

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Nevertheless, oxidative stress occurs when active oxygen synthesis surpasses the antioxidant potential in the body[6, 7]. Oxidative stress reduces the number of gametes and sperm motility by increasing the proportion of dead cells. For sperm membrane protection from oxidative damage, a highly efficient antioxidant mechanism is required[8].

Bioflavonoid hesperetin is 5, 7, 30 - trihydroxy -40- methoxy flavanone, widely used in Chinese medicine[9]. It is commonly present in citrus plants as the hesperidin glycoside employed as a prodrug[10]. Intestinal microorganisms convert dietary hesperidins to hesperetin before absorption[11]. The anti-inflammatory, antioxidant, antibacterial, anticarcinogenic, and anti-allergic activities of hesperetin have been demonstrated[12]. It is an effective antioxidant against cadmium-induced testicular damage[13]. In addition, hesperetin shows a protective effect against doxorubicin-induced cardiotoxicity[14]. Hesperetin can also prevent the testicular toxicity caused by doxorubicin and minimize the testicular injury risk in diabetic mice by decreasing oxidative stress, apoptosis, and inflammation[15].

In avian spermatozoa, the addition of antioxidants to freeze extenders has been tested in several experiments. Furthermore, by using antioxidants during sperm thawing, several changes were made to reduce the incidence of oxidative stress[16].

The current study aims to investigate the effects of varying concentrations of hesperetin on the quality of the rooster's semen during cryopreservation.

2. Materials and Methods

Unless otherwise specified, Merck was the supplier of all materials (Darmstadt, Germany). This experiment was conducted with the agreement of the Animal Science Department at the University of Tabriz.

2.1 Animals and Sample Preparation

Ten 28-week-old Broiler roosters were kept in separate enclosures (70 x 95 x 85) cm maintained at (18 to 20) °C and treated to a (15 L: 9 D) photoperiod. To feed the birds, commercial feed for breeding flocks was utilized. All the time, water was available ad libitum.

To collect sperm, a dorso-abdominal massage is used[5]. The dorso-abdominal was used twice a week for semen collection. They were placed in a 37°C water bath immediately. 5 ejaculates per 10 cocks were prepared for semen analysis. Ejaculated sperm motility of 80%, sperm concentration of 3 x 10^9 sperm/ml, volume between 0.2 and 0.6 ml, and 10% irregular morphology were combined and divided into 6 portions for D-fructose dilution. (8 g/L) Lake-extender, polyvinylpyrrolidone (3 g/L), 19.2 g/L sodium glutamate, glycine (3.74 g/L), 0.7 g/L, pH 7.1 magnesium acetate, potassium citrate (5 g/L) and osmolarity of 310 mOsm/kg. At 37°C, sperm were stretched in Lake Extender with various concentrations of hesperetin (control (0), 15, 20, 25, and 30 µM). Soon after diluting, the processed samples were drawn with polyvinyl into straws (0.25) mL and then equilibrated at 4 °C for 3 h[17]. After achieving equilibrium, samples were on a liquid nitrogen surface (7 minutes) before submerging. After a week, they were individually thawed in a water bath (37.8°C/30 sec).

2.2 Motility Analysis

A sperm analyzer (CASA) system IVOS 12 was used for sperm characterization assay. After diluting and thawing the samples, three millilitres of sperm were placed on a prewar med chamber slide. The samples were examined using at least 200 cells. The total motility (TM %), progressive motility (PM, %), velocity average path (VAP, mm/s), curvilinear velocity (VCL, mm/s), straight-line velocity (VSL, mm/s), straightness (STR, %) and linearity (LIN, %) were assessed[18].

2.3 Plasma Membrane

10 µL of thawed frozen sample was treated with hypo-osmotic solution (100) mL (100 mOsmol/kg sodium-citrate at 1.9 µM and fructose at 5.0 µM in 100 mL of distilled water) for 30 minutes to evaluate the plasma membrane of rooster spermatozoa[19]. Two hundred spermatozoa were examined under a 400x microscope for damaged and intact plasma membranes.

2.4 Sperm Morphological Abnormalities

10 ml of sperm solution was deposited in Eppendorf tubes with one milliliter of Hancock solution to evaluate sperm morphological abnormalities. Aberrant sperm total number was recorded using a microscope on 200 sperm per experimental treatment and duplicate[20].

2.5 Phosphatidylserine Externalization (Annexin V/PI)

The phosphatidylserine detection kit was utilized to identify the PS flip-flop movement across the membrane. The cell concentration was readjusted to sperm / mL (1 x 10^6) by washing the samples in 100 µL calcium buffer. Subsequently, 10 µL of Annexin V FITC was added and incubated (24°C / 20 min). The preceding cell solution was then combined with 10 µL PI and incubated at 24°C for 20 min. Used flow cytometry to label sperm instantly by fluorescence emission measuring at 530/30 nm FL1 canal and 585/42 FL3 canal. Early sperm apoptosis were labeled (An+/PI-), viable sperm label (An-\PI-), necrotic sperm were labeled (An-\PI+), and apoptotic sperm were labeled (An+\PI+)[21].

2.6 Mitochondrial Activity

The Rhodamine-123 (RH-123) assay was applied for evaluating sperm mitochondrial activity. Thawed diluted sperm (250 µL) 5 µL of (0.01, 1) mg\mL stock R123 and PI solution were inputted. Before flow cytometry, incubated samples at 37°C for 15 min. For determining the mitochondrial activity, the percentage of sperm with R123 high fluorescence and no PI fluorescence was recorded[22].

2.7 Malondialdehyde (MDA) Concentrations

As a biomarker for lipid peroxidation, thiobarbituric-acid reaction was used for determining MDA. Protein precipitate was obtained by adding cold trichloroacetic acid (1mL, 20% wt/vol) to diluted sperm (1mL). The pellets were then formed by centrifugation (963 X g / 15 min), and the supernatant (1 mL) was removed and treated in a water bath (100°C / 10 minutes) with thiobarbituric-acid (1 mL, 0.67% wt. /vol.)[23]. Then they were cooled, and their absorbance at 532 nm was measured with a spectrophotometer.

2.8 Statistical Analysis

The Shapiro-Wilk test and the UNI-VARIATE technique were used to evaluate the normal distribution. For data analysis, the SAS software's MIXED technique (SAS Institute V 9.1, 2002, Cary, NC) was chosen. Hesperetin levels' fixed effects were incorporated in the statistical model. The results were stated as Lsmeans $\pm$ SEM with Tukey's test to define the significant differences. The differences with P values < 0.01 were considered significant, it should be mentioned.

3. Results

Tables 1, 2, and 3 illustrate the effect of hesperetin levels on abnormal morphology, motility characteristics, membrane integrity, mitochondrial activity, and apoptotic status of frozen sperm. In comparison to the control (0), hesperetin significantly increased progressive motility% (PM%), straight-line velocity

mm/s (VSL mm/s), total motility% (TM%), beat cross frequency Hz (BCF Hz), velocity average path mm/s (VAP mm/s), velocity curvilinear mm/s (VCL mm/s), linearity% (LIN%), straightness% (STR%), membrane integrity%, mitochondrial activity %. It was significantly lower for the lateral head displacement amplitude mm (ALH mm), total abnormality %, dead sperm %, and apoptotic sperm % compared to the control. However, at level 30, the hesperetin effect was reversed for all parameters.

Table 1: Effect of different levels of hesperetin on motility parameters of rooster sperm.

Treatments (mg/L)	TM (%)	PM (%)	VAP (mm/s)	VSL (mm/s)	VCL (mm/s)	LIN (%)	STR (%)	ALH (mm)	BCF (Hz)
Control (0)	40.12^{b}	16.59^{c}	28.85^{c}	17.03^{b}	53.78^{a}	32.26^{a}	59.10^{a}	4.52^{a}	14.09^{a}
15	44.76^{b}	18.32^{bc}	30.45^{bc}	17.88^{b}	54.48^{a}	33.09^{a}	59.41^{a}	4.40^{a}	14.84^{a}
20	57.95^{a}	21.93^{b}	33.42^{ab}	20.4^{ab}	58.49^{a}	34.41^{a}	60.37^{a}	4.17^{a}	15.70^{a}
25	64.26^{a}	30.44^{a}	35.88^{a}	21.77^{a}	61.08^{a}	35.66^{a}	61.14^{a}	3.94^{a}	16.23^{a}
30	42.54^{b}	17.37^{c}	29.78^{bc}	17.13^{b}	53.98^{a}	31.74^{a}	58.11^{a}	4.67^{a}	14.69^{a}
SEM	1.56	1.02	1.03	0.82	1.80	1.74	3.44	0.22	1.36

Abbreviations: ALH, mean amplitude of the lateral head displacement; BCF, mean of the beat cross frequency; LIN, linearity; PM, progressive motility; STR, straightness; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity, and SEM, Standard Error.

$^{a-c}$ Different superscripts within the same column indicate group differences ($P < 0.01$).

There were significant differences in progressive motility% (PM %), straight-line velocity mm/s (VSL mm/s), total motility% (TM %), membrane integrity%, velocity average path mm/s (VAP mm/s), mitochondria activity%, apoptotic sperm (%), and live sperm% after hesperetin treatment. There is no statistically significant difference between straightness% (STR %), curvilinear velocity mm/s (VCL mm/s), linearity% (LIN %), lateral head-displacement amplitude mm (ALH mm), total abnormality%, beat cross frequency Hz (BCF Hz), and dead sperm%.

Tables 1, 2, and 3 show no significant difference between levels 20 and 25 for all parameters except progressive motility %. There was no significant difference between controls (0), level 15, and level 30.

Table 2: Effect of different levels of hesperetin on membrane integrity, total abnormality, and mitochondria activity of rooster sperm.

Treatments (mg/L)	Membrane integrity (%)	Total abnormality (%)	Mitochondria activity (%)
Control (0)	38.36^{b}	17.29^{a}	36.90^{b}
15	41.10^{b}	17.17^{a}	41.01^{b}
20	56.53^{a}	14.16^{a}	56.84^{a}
25	61.71^{a}	13.67^{a}	61.71^{a}
30	41.26^{b}	17.19^{a}	38.79^{b}
SEM	1.82	1.33	2.02

$^{a-c}$ Different superscripts within the same column indicate group differences ($P < 0.01$).

It was confirmed that hesperetin level 25 µM recorded the highest values (64.26%, 30.44%, 35.88 mm/s, 21.77 mm/s, 61.08 mm/s, 35.66%, 61.14%, 16.23 Hz, 61.71%, 61.71% and 61.33%) with VCL mm/s, VAP mm/s, TM%, PM%, VSL mm/s, LIN%, STR%, BCF Hz, membrane integrity%, mitochondria activity% and live sperm%, respectively). In contrast, it recorded the lowest values (3.94mm, 13.67%, 13.30% and 25.37%) with ALH mm, total abnormality%, apoptotic sperm% and dead sperm %, respectively ($P < 0.01$). Nevertheless, total abnormality% and apoptotic sperm% recorded the highest values ($P < 0.01$) (17.29% and 29.13%, respectively) with control (0), and the lowest values (13.67% and 13.30%, respectively) were recorded with level 25 of hesperetin.

Table 3: Effect of different levels of hesperetin on live, apoptosis, and dead status of rooster sperm after freeze-thawing.

Treatments(mg/L)	Live sperm %	Apoptotic sperm %	Dead sperm %
Control (0)	38.91^{b}	29.13^{a}	31.95^{a}
15	43.78^{b}	28.79^{a}	27.43^{a}
20	56.04^{a}	17.50^{b}	26.46^{a}
25	61.33^{a}	13.30^{b}	25.37^{a}
30	39.01^{b}	28.88^{a}	32.11^{a}
SEM	1.49	1.38	2.18

$^{a-c}$ Different superscripts within the same column indicate group differences ($P < 0.01$

4. Discussion

This study was conducted to evaluate deferent levels of an antioxidant that is known hesperetin, it can be used to increase

the sperm quality. We tested different hesperetin concentrations to achieve this goal.

The results obtained from the flow cytometric analysis in the current research showed the hesperetin beneficial effects on structure and quality cryopreserved sperm cell. After thawing, the sperm mitochondrial activity was higher in treatments containing medium concentration of hesperetin. It was demonstrated that hesperetin treatment can minimize the deleterious effects of cryopreservation on spermatozoa, hence increasing sperm fertility potential following the freezing-thawing technique. This research shows that hesperetin increased spermatozoa motility, morphology, and viability. These findings are consistent with earlier results[5], which shows that during cryopreservation antioxidants can stabilize plasma membranes and mitochondria in rooster semen from oxidative stress[16].

Moreover, hesperetin can lower reactive oxygen species (ROS) levels and the ratio of sperm apoptosis. Hesperetin used in traditional medicine, and it is a bioflavonoid[9]. It is present in citrus as the glycoside, which functions as a prodrug[10]. Hesperetin can lower the ratio of DNA damage, sperm head abnormalities, and MDA generated by doxorubicin, it was discovered recently[14]. In one study, hesperetin was found to lower ROS in diabetic rats, as well as oxidative stress, apoptosis, and inflammation[15]. Recent research revealed high hesperetin concentrations trigger death via the mitochondrial pathway[24].

During cryopreservation, the sperm antioxidant level falls[25]. Recent research has demonstrated that adding antioxidants to freezing media can increase sperm quality after cryopreservation[16]. The hesperetin concentration of 20 µM considerably improved the VSL, VAP, sperm velocity parameters, and VCL[26].

The findings of our study showed that the 20 and 25 µM hesperetin significantly decreased ROS generation.

Recent research has demonstrated that hesperetin possesses significant antioxidant properties; the findings of this investigation indicate that hesperetin possesses potent antioxidant properties[14, 15, 26].

Conclusions

According to our observations, the spermatozoa treatment with 25 µM hesperetin in the sperm extender improved sperm quality the most the other treatments, and it to the semen extender appears to be an effective method for preserving the quality of cryopreserved rooster sperm. In addition, hesperetin's protective properties against oxidative stress and ability to mitigate the adverse effects of the post-thawing procedure on sperm quality are noteworthy.

Conflict of interests

None

Authors contribution

The authors made equal contributions throughout the entire process, encompassing everything from designing and executing the research to analyzing the findings and composing the manuscript.

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References

1. Hanafy, A. M., & Elnesr, S. S. Induction of reproductive activity and egg production by gonadotropin-releasing hormone in non-laying hens. *Reproduction in Domestic Animals* **56(9)**, 1184-1191, doi: 10.1111/rda.13972 (2021).
2. Mehaisen, G. M., Partyka, A., Ligocka, Z., & Niżański, W. Cryoprotective effect of melatonin supplementation on post-thawed rooster sperm quality. *Animal reproduction science* **212**, 106238, doi: 10.1016/j.anireprosci.2019.106238 (2020).
3. Al-Qarawi, A. A. Infertility in the dromedary bull: a review of causes, relations and implications. *Animal reproduction science* **87(1-2)**, 73-92, doi: 10.1016/j.anireprosci.2004.11.003 (2005).
4. Nabi, F., Arain, M. A., Rajput, N., Alagawany, M., Soomro, J., Umer, M., ... & Liu, J. Health benefits of carotenoids and potential application in poultry industry: A review. *Journal of animal physiology and animal nutrition* **104(6)**, 1809-1818, doi: 10.1111/jpn.13375 (2020).
5. Najafi, A., Kia, H. D., Mehdipour, M., Hamishehkar, H., & Álvarez-Rodríguez, M. Effect of quercetin loaded liposomes or nanostructured lipid carrier (NLC) on post-thawed sperm quality and fertility of rooster sperm. *Theriogenology* **152**, 122-128, doi: 10.1016/j.theriogenology.2020.04.033 (2020).
6. Elnesr, S. S., Elwan, H. A. M., Xu, Q. Q., Xie, C., Dong, X. Y., & Zou, X. T. Effects of in ovo injection of sulfur-containing amino acids on heat shock protein 70, corticosterone hormone, antioxidant indices, and lipid profile of newly hatched broiler chicks exposed to heat stress during incubation. *Poultry Science* **98(5)**, 2290-2298, doi: 10.3382/ps/pey609 (2019).
7. Elwan, H. A., Elnesr, S. S., Xu, Q., Xie, C., Dong, X., & Zou, X. Effects of in ovo methionine-cysteine injection on embryonic development, antioxidant status, IGF-I and TLR4 gene expression, and jejunum histomorphometry in newly hatched broiler chicks exposed to heat stress during incubation. *Animals* **9(1)**, 25, doi: 10.3390/ani9010025 (2019).
8. Mavi, G. K., Dubey, P. P., & Cheema, R. S. Association of antioxidant defense system with semen attributes vis a vis fertility in exotic and indigenous chicken breeds. *Theriogenology* **144**, 158-163, doi: 10.1016/j.theriogenology.2020.01.003 (2020).
9. Gil-Izquierdo, A., Gil, M. I., Ferreres, F., & Tomás-Barberán, F. A. In vitro availability of flavonoids and other phenolics in orange juice. *Journal of Agricultural and Food Chemistry* **49(2)**, 1035-1041, doi: 10.1021/jf0000528 (2001).
10. Lee, N. K., Choi, S. H., Park, S. H., Park, E. K., & Kim, D. H. Antiallergic activity of hesperidin is activated by intestinal microflora. *Pharmacology* **71(4)**, 174-180, doi: 10.1159/000078083 (2004).
11. Kim, H. K., Jeong, T. S., Lee, M. K., Park, Y. B., & Choi, M. S. Lipid-lowering efficacy of hesperetin metabolites in high-cholesterol fed rats. *Clinica chimica acta* **327(1-2)**, 129-137, doi: 10.1016/S0009-8981(02)00344-3 (2003).
12. Fernández-Rojas, B., & Gutiérrez-Venegas, G. Flavonoids exert multiple periodontic benefits including anti-inflammatory, periodontal ligament-supporting, and alveolar bone-preserving effects. *Life sciences* **209**, 435-454, doi: 10.1016/j.lfs.2018.08.029 (2018).
13. Shagirtha, K., & Pari, L. Hesperetin, a citrus flavonone, protects potentially cadmium induced oxidative testicular dysfunction in rats. *Ecotoxicology and environmental safety* **74(7)**, 2105-2111, doi: 10.1016/j.ecoenv.2011.06.002 (2011).
14. Trivedi, P. P., Kushwaha, S., Tripathi, D. N., & Jena, G. B. Cardioprotective effects of hesperetin against doxorubicin-induced oxidative stress and DNA

damage in rat. *Cardiovascular toxicology* **11(3)**, 215-225, doi: 10.1007/s12012-011-9114-2 (2011).

15. Samie, A., Sedaghat, R., Baluchnejadmojarad, T., & Roghani, M. Hesperetin, a citrus flavonoid, attenuates testicular damage in diabetic rats via inhibition of oxidative stress, inflammation, and apoptosis. *Life sciences* **210**, 132-139, doi: 10.1016/j.lfs.2018.08.074 (2018).

16. Salih, S. A., Daghigh-Kia, H., Mehdipour, M., & Najafi, A. Does ergothioneine and thawing temperatures improve rooster semen post-thawed quality?. *Poultry Science* **100(10)**, 101405, doi: 10.1016/j.psj.2021.101405 (2021).

17. Mehdipour, M., Daghigh-Kia, H., Najafi, A., & Martínez-Pastor, F. Type III antifreeze protein (AFP) improves the post-thaw quality and in vivo fertility of rooster spermatozoa. *Poultry science* **100(8)**, 101291, doi: 10.1016/j.psj.2021.101291 (2021).

18. Najafi, A., Kia, H. D., Mehdipour, M., Shamsollahi, M., & Miller, D. J. Does fennel extract ameliorate oxidative stress frozen-thawed ram sperm?. *Cryobiology* **87**, 47-51, doi: 10.1016/j.cryobiol.2019.02.006 (2019).

19. Mehdipour, M., Kia, H. D., Moghaddam, G., & Hamishehkar, H. Effect of egg yolk plasma and soybean lecithin on rooster frozen-thawed sperm quality and fertility. *Theriogenology* **116**, 89-94, doi: 10.1016/j.theriogenology.2018.05.013 (2018).

20. Najafi, A., Taheri, R. A., Mehdipour, M., Martínez-Pastor, F., Rouhollahi, A. A., & Nourani, M. R. Improvement of post-thawed sperm quality in broiler breeder roosters by ellagic acid-loaded liposomes. *Poultry science* **98(1)**, 440-446, doi: 10.3382/ps/pey353 (2019).

21. Najafi, A., Taheri, R. A., Mehdipour, M., Farnoosh, G., & Martínez-Pastor, F. Lycopene-loaded nanoliposomes improve the performance of a modified Beltsville extender broiler breeder roosters. *Animal reproduction science* **195**, 168-175, doi: 10.1016/j.anireprosci.2018.05.021 (2018).

22. Mehdipour, M., Daghigh Kia, H., Najafi, A., Mohammadi, H., & Álvarez-Rodriguez, M. Effect of crocin and naringenin supplementation in cryopreservation medium on post-thaw rooster sperm quality and expression of apoptosis associated genes. *PLoS One* **15(10)**, e0241105, doi: 10.1371/journal.pone.0241105 (2020).

23. Mehdipour, M., Kia, H. D., Najafi, A., Dodaran, H. V., & García-Álvarez, O. Effect of green tea (Camellia sinensis) extract and pre-freezing equilibration time on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology* **73(3)**, 297-303, doi: 10.1016/j.cryobiol.2016.10.008 (2016).

24. Zhang, J., Song, J., Wu, D., Wang, J., & Dong, W. Hesperetin induces the apoptosis of hepatocellular carcinoma cells via mitochondrial pathway mediated by the increased intracellular reactive oxygen species, ATP and calcium. *Medical Oncology* **32**, 1-11, doi: 10.1007/s12032-015-0516-z (2015).

25. Len, J. S., Koh, W. S. D., & Tan, S. X. The roles of reactive oxygen species and antioxidants in cryopreservation. *Bioscience reports* **39(8)**, doi: 10.1042/BSR20191601 (2019).

26. Valipour, J., Mojaverrostami, S., Abouhamzeh, B., & Abdollahi, M. Protective effects of hesperetin on the quality of sperm, apoptosis, lipid peroxidation, and oxidative stress during the process of cryopreservation: An experimental study. *International Journal of Reproductive BioMedicine* **19(1)**, 35, doi: 10.18502/ijrm.v19i1.8178 (2021).