



Short Communication

Oxidative Stress Induction by Infectious Pancreatic Necrosis Virus in RTG-2 Cell Line

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ABSTRACT

Investigation of the relationship between oxidative stress and cell damage may provide important insight into infection treatment. Previous studies have shown that many viruses induce oxidative cell damage during their replication in mammalian cell culture, but fish viruses has not been well-studied. Infectious pancreatic necrosis (IPN) is caused by infectious pancreatic necrosis virus (IPNV) and it is a viral, fatal and contagious disease of young salmonids and nonsalmonid fish with acute to peracute clinical forms. This study was designed to investigate the relationship between oxidative stress and cell damage due to IPNV in rainbow trout gonad cell line-2 (RTG-2). For this purpose, IPNV was inoculated in RTG-2. Then, media of IPNV infected cell and media of negative control were collected (n=3) for each incubation time (0, 8, 24, 48, 72 and 96 h) and superoxide dismutase (SOD), glutathione peroxidase (GPx) activities and malondialdehyde (MDA) concentrations were measured by the spectrophotometric methods. Data obtained revealed higher MDA levels in IPNV infected cells than negative control medium (p<0.001). However, SOD and GPx levels in IPNV infected cells were lower than negative control cells medium (p<0.001). These results indicate that, cell damage in RTG-2 cell line, that is caused by IPNV is associated with oxidative stress.

Article Information

Received 26 December 2016

Revised 14 June 2020

Accepted 11 March 2020

Available online 10 June 2021

(early access)

Published 05 March 2022

Authors' Contributions

SG contributed in experiment design. GFY, CT and AS analyzed the data. SG, GFY statistically analyzed the data and wrote the article. GI helped in data acquisition, literature research and discussion.

Key words

IPNV, RTG-2, Oxidative stress.

The rainbow trout farming industry is one of the largest fish aquaculture industries in Turkey. Infectious pancreatic necrosis (IPN) is a viral, fatal and contagious disease of young salmonids and nonsalmonid fish with acute to peracute clinical forms. It is caused by IPN virus (IPNV), which is a member of the genus *Aquabirnavirus*, family Birnaviridae (Bowers *et al.*, 2008).

More reactive oxygen species (ROS) are produced by eukaryotic cells using oxygen in order to produce energy for normal metabolic activities. These free radicals which are produced during normal metabolism are neutralized by antioxidants to prevent oxidative stress which occurs in the cell (Halliwell, 1994). In the presence of oxidative stress, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase constitutes less toxic metabolites of ROS (Morales *et al.*, 2004; Romero *et al.*, 2011) and increased MDA concentrations and decreased SOD and GPx activities clearly indicates the presence of

oxidative stress (Duracková, 2010; Reed, 1995).

Many studies have shown that many of viruses induce oxidative cell damage due to their replication (Gullberg *et al.*, 2015; Muller, 1992; Najafi *et al.*, 2014; Peterhans *et al.*, 1987; Vierucci *et al.*, 1983). Previous studies have determined that mammalian RNA viruses (Peterhans *et al.*, 1987), DNA viruses (Vierucci *et al.*, 1983) and retroviruses (Muller, 1992) can stimulate oxidative stress and lead to cell death. Investigations on oxidative stress and cell damage by fish viruses were reported only for Betanodavirus (Chang *et al.*, 2011), hence relationship of IPNV (member of the genus *Aquabirnavirus*) and oxidative stress in cell culture had not been investigated until now. The objective of the present study is to evaluate the oxidative stress-induced cell damage by IPNV in RTG-2 cell line.

Materials and methods

RTG-2 cell lines were used for IPNV inoculation in this study. RTG-2 cells were cultured into Eagle Minimal Essential Medium (EMEM) and supplemented with 10% fetal calf sera and incubated at 28°C. When the cells

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0030-9923/2022/0003-1459 \$ 9.00/0

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reached about 80% confluence, the cells were infected with IPNV (MOI:1) and incubated at 28°C. Also, EMEM was inoculated in negative control. Virus inoculated cell flasks were checked daily under the invert microscope to determine an IPNV cytopathic effect presence. Virus and cell control media were sampled (n=3) for each time period 0, 8, 24, 48, 72 and 96 h and stored at +4°C until analysed for biochemical parameters of oxidative stress. Superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA) concentrations were analysed in all of the medium samples.

MDA in cell culture was determined according to the method reported by Yoshioka *et al.* (1979). Briefly, 0.5 ml cell medium, 2.5 ml, 20% of trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid were added into test tube. 500 µl trichloroacetic acid was added in blank tube. Tubes were shaken by vortex and heated at 90°C for 30 min. Then, 4.0 ml of n-butanol was added and centrifuged at 1550 g for 10 min. The upper butanol phase was removed and absorbance was recorded at 535 nm at spectrophotometer and MDA concentration was determined according to the standard curve.

The measurement of SOD activity was performed by spectrophotometric method (Sun *et al.*, 1989). Conditioned cell medium (100 µl) was added in a mixture containing 2.0 ml chloroform/ethanol (3:5/v:v) and centrifuged at 4°C, 1550 g for 15 min. Amount of 0.5 ml chloroform phase was aspirated and 2.45 ml working reagent (0.3 mM/l xanthine 0.6 mM/l Na₂EDTA, 150 µmol/l NBT, 400 mmol/l Na₂CO₃, 1g/l BSA) + 0.5 ml bidistilled water + 50 µl xanthine oxidase (167 U/l) was added. 0.5 ml bidistilled water was added in blank tube instead of cell medium. Tubes were placed in water bath and incubated at 25°C for 20 min. Then, reaction was stopped by adding 1.0 ml 0.8 mmol/l of copper (II) chloride and centrifuged at 1550 g for 10 min. The absorbance of supernatants was measured at 560 nm and enzyme activity was calculated.

The activity of GPx was measured by the method of Paglia and Valentine (1967). GPx activity was measured in

20 µL of conditioned cell medium in a mixture containing 100 µl glutathione, 100 µl nicotinamide adenine dinucleotide phosphate (NADPH), 10 µl glutathione reductase, 10 µl sodium azide (NaN₃) placed on ice. Tubes were shaken vigorously by vortex. Reaction was started with the addition of 100 ml H₂O₂ and the mixture was incubated at room temperature for 30 min. After the reaction was stopped, the GPx consumption was measured. Its absorbance at 340 nm was read spectrophotometrically. GPx was calculated according to one unit of GPx activity, which is defined as the amount of enzyme required to oxidize 1 mmol of NADPH /min.

SPSS for Windows (version 22) and R studio for Windows (version 3.0.2) was used for statistical analyses. MDA concentrations, SOD and GPx activities of IPNV and control were analyzed with relation to time by generalized linear models. A value of p<0.001 was regarded to indicate a statistically significant difference between group mean values.

Results and discussion

In this study, the repeated measurements (n=3) of MDA concentrations for IPNV cultivated cells media at 0, 8, 24, 48, 72 and 96 h, significantly (p<0.001) increased after the 48th h, SOD concentrations were significantly (p<0.001) decreased after 48th h and GPx concentrations were significantly (p<0.001) decreased after 8th h, compared to control cells (Table 1).

Oxidative stress arising from excessive ROS can lead to cell damage and death by suppressing the defense mechanisms of the cells and oxidative degradation of the macromolecules (Ryter *et al.*, 2007). Antioxidant enzymes play an important role in protecting cells against oxidative stress by scavenging of excessively produced ROS (Halliwell, 1994). Oxidative stress occurs when the ROS generation is beyond the capacity of antioxidant enzymes (Duracková, 2010; Reed, 1995). Damage due to ROS is inevitable for all creatures that breathe oxygen. Oxidative stress and antioxidant defense mechanisms in

Table I.- Effect of infection RGT-2 cells with IPNV on MDA, SOD and GPx levels after different time intervals.

Time	MDA (nmol/ml)			SOD (U/ml)			GPx (U/ml)		
	Control	Virus	Sig.**	Control	Virus	Sig.**	Control	Virus	Sig.**
0 h	1.99 ± 0.19	2.18 ± 0.39	0.257	43.42 ± 2.13	45.57 ± 3.32	0.501	0.34 ± 0.00	0.32 ± 0.01	0.063
8 h	1.84 ± 0.06	2.17 ± 0.42	0.039	41.57 ± 2.13	45.93 ± 4.54	0.171	0.33 ± 0.01	0.29 ± 0.00	<0.001*
24 h	2.03 ± 0.13	2.25 ± 0.39	0.174	45.76 ± 4.47	46.18 ± 6.00	0.894	0.32 ± 0.00	0.28 ± 0.01	<0.001
48 h	1.91 ± 0.12	3.10 ± 0.11	<0.001*	43.17 ± 2.55	28.84 ± 6.66	<0.001*	0.31 ± 0.01	0.16 ± 0.02	<0.001
72 h	1.95 ± 0.06	3.02 ± 0.22	<0.001	46.73 ± 5.96	28.10 ± 6.54	<0.001	0.29 ± 0.00	0.14 ± 0.02	<0.001
96 h	1.99 ± 0.00	3.02 ± 0.27	<0.001	45.45 ± 5.00	26.19 ± 4.88	<0.001	0.28 ± 0.01	0.12 ± 0.01	<0.001

*Statistically significant according to the generalized linear models test P<0.05. **The samples were tested in triplicate, and the data were presented in mean Sig. This experiment was repeated three times with similar results.

fish have been investigated (Bayir *et al.*, 2011), but, the role of oxidative stress in cellular damage caused by IPNV has not been studied before. The results of this study revealed that cell damage of RTG-2 cell line is caused by IPNV related to oxidative stress.

It is already reported that IPNV infection causes economic losses in the production of rainbow trout (Bowers *et al.*, 2008; Espinoza *et al.*, 2005; Albayrak and Ozan, 2010; Candan, 2002). The association of apoptosis and necrotic changes have already been reported with IPNV infection in culture (Hong *et al.*, 1998; Espinoza *et al.*, 2005), but little is known about relationship of this infection with oxidative stress damage. In this study, the effect IPNV infection on oxidative stress damage has been studied. At the end of the biochemical analyses, MDA concentrations of IPNV produced cell media were significantly ($p < 0.001$) increased from the 48th h, SOD and GPx activities were significantly ($p < 0.001$) decreased from the 48th and 8th h, respectively, compared to control cell medium.

In conclusion, this study revealed that cell damage caused by IPNV in cell culture is related to oxidative stress for the first time and antioxidants may be an option in treatment of this disease.

Acknowledgement

This study was supported by Research Fund of Ondokuz Mayıs University Samsun/Turkey (Project No. PYO.VET.1904.15.008).

Statement of conflict of interest

The authors have declared no conflict of interest.

References

- Albayrak, H. and Ozan, E., 2010. *Ankara Üniv. Vet. Fak. Derg.*, **57**: 125-129. https://doi.org/10.1501/Vetfak_0000002322
- Bayir, A., Sirkecioglu, A.N., Bayir, M., Aras, N.M., Haliloglu, H.I., Kocaman, E.M. and Aras, M.N., 2011. *Comp. Biochem. Physiol.*, **159**: 191-196. <https://doi.org/10.1016/j.cbpb.2011.04.008>
- Bowers, R.M., Lapatra, S.E. and Dhar, A.K., 2008. *J. Virol. Methods*, **147**: 226-234. <https://doi.org/10.1016/j.jviromet.2007.09.003>
- Candan, A., 2002. *Bull. Eur. Assoc. Fish Pathol.*, **22**: 45-47.
- Chang, C.W., Yu-Chin, S., Guor-Mour, H., Chuian-Fu, K. and Jiann-Ruey, H., 2011. *PLoS One*, **6**: e25853. <https://doi.org/10.1371/journal.pone.0025853>
- Duracková, Z., 2010. *Physiol. Res.*, **59**: 459-69.
- Espinoza, J.C., Cortes-Gutierrez, M. and Kuznar, J., 2005. *Virus Res.*, **109**: 133-138. <https://doi.org/10.1016/j.virusres.2004.10.014>
- Gullberg, R.C., Jordan-Steel, J., Stephanie, L., Moon, E., Inaz, S. And Geiss, B.J., 2015. *Virology*, **475**: 219-229. <https://doi.org/10.1016/j.virol.2014.10.037>
- Halliwell, B., 1994. *Nutr. Rev.*, **52**: 253-265. <https://doi.org/10.1111/j.1753-4887.1994.tb01453.x>
- Hong, J.R., Lin, T.L., Hsu, Y.L. and Wu, J.L., 1998. *Virology*, **250**: 76-84. <https://doi.org/10.1006/viro.1998.9347>
- Morales, A.E., Pérez-Jiménez, A., Hidalgo, M.C., Abellán, E. and Cardenete, G., 2004. *Comp. Biochem. Physiol.*, **139**: 153-161. <https://doi.org/10.1016/j.cca.2004.10.008>
- Muller, F., 1992. *Free Radic. Biol. Med.*, **13**: 651-657. [https://doi.org/10.1016/0891-5849\(92\)90039-J](https://doi.org/10.1016/0891-5849(92)90039-J)
- Najafi, A., Salati, A.P., Yavari, V. and Asadi, F., 2014. *Int. J. aquat. Biol.*, **2**: 246-252.
- Paglia, D.E. and Velentine, W.N., 1967. *J. Lab. clin. Med.*, **70**: 158-169.
- Peterhans, E., Grob, M., Burge, T. and Zanoni, R., 1987. *Free Radic. Res. Commun.*, **3**: 39-46. <https://doi.org/10.3109/10715768709069768>
- Reed, D.J., 1995. In: *Molecular and cellular mechanisms of toxicity* (eds. F. de Matteis and L.L. Smith). CRC Press, Fla, USA, pp. 35-68.
- Ryter, S.W., Kim, H.P., Hoetzel, A., Park, J.W., Nakahira, K., Wang, X. and Choi, A.M., 2007. *Antioxid. Redox. Signal.*, **9**: 49-89. <https://doi.org/10.1089/ars.2007.9.49>
- Romero, M.C., Tapella, F., Sotelano, M.P., Ansaldo, M. and Lovrich, G.A., 2011. *Aquaculture*, **319**: 205-210. <https://doi.org/10.1016/j.aquaculture.2011.06.041>
- Sun, Y., Oberley, L.W., Elwell, J.H. and Sierra-Rivera, E., 1989. *Int. J. Cancer*, **44**: 1028-1033. <https://doi.org/10.1002/ijc.2910440615>
- Vierucci, A., DeMartino, M., Graziani, E., Rossi, M.E. and London, W.T., 1983. *Pediatr. Res.*, **10**: 814-820. <https://doi.org/10.1203/00006450-198310000-00010>
- Yoshioka, T., Kawada, K., Shimada, T. and Mori, M., 1979. *Am. J. Obstet. Gynecol.*, **135**: 372-376. [https://doi.org/10.1016/0002-9378\(79\)90708-7](https://doi.org/10.1016/0002-9378(79)90708-7)