

## Increased Hsp-72 Expression in Oral Mucormycosis after treatment with Hyperbaric Oxygen (HBO)

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### Abstract

Mucormycosis is a rare invasive fungal infection, but fatal when it occurs and triggered by tooth extraction. Heat Shock Protein (HSP)-72 allegedly serves to protect the cell from stress.

The aim of this study is to analyze the expression of Hsp-72 in maxillary mucosal tissue of mucormycosis infection on dental extraction after Hyperbaric Oxygen (HBO) therapy. 34 male marmots with age of 3-4 month were divided into 6 groups: 1 control group, 1 group of dental extraction and 4 intervention group. Two group, first with injection of *Rhizopus oryzae* strain CBS 110.17, and then tooth extraction. The other two group, dental extraction first and then *rhizopus oryzae* injection. The four interventions group were divided with non HBO and HBO treatment of 2,4 ATA 3x30 minute, one session, for 5 days. The expression of HSP-72 was examined by immunohistochemistry. Mean of (K-1): 0,17±0,12; (K-2): 0,62±0,15; (K-3): 0,52±0,19; (K-4): 6,07±2,79; (K-5): 1,18±0,82; and (K-6): 2,92±0,84. K-4 is the most dominant compared to the other group.

HBO therapy 2,4 ATA, 3x30', for 5 days has been significantly proven to increase the expression of Hsp-72 in macrophage of maxillary mucosal which infected by mucormycosis until 35,7 times higher than normal group.

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### Introduction

Mucormycosis is a rare opportunistic infection and was first described by Paultauf in 1885. It is recognized as one of the most rapidly progressive lethal form of fungal infection in human beings with a high mortality of 70-100%. The most commonly reported form of the disease is rhinocerebral mucormycosis, which is characterized by progressive fungal invasion of the hard palate, paranasal sinuses, orbit, and brain. It can be manifests as rhinocerebral,

pulmonary, gastrointestinal, cutaneous or disseminated form. The conditions predisposing to mucormycosis are diabetes mellitus, renal failure, tuberculosis, organ transplant, longterm corticosteroid, immunosuppressive therapy, cirrhosis, burns, protein energy malnutrition, leukemias, lymphomas and AIDS.<sup>1</sup> Oral manifestation of these infections occur when the organism enter through an invasive portal such as a dental extraction.<sup>2</sup> Successful management of this fulminant infection requires early recognition of the disease and aggressive medical and surgical intervention to prevent the high morbidity and mortality associated with the disease process. Mucormycosis is the third invasive mycosis in order of importance after candidiasis and aspergillosis and is caused by the fungi class of Zygomycetes. The most

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important species in order of frequency is *Rhizopus arrhizus (oryzae)*.<sup>3</sup> Mucormycosis is a rare opportunistic fungal infection which affects less than two people in a million.<sup>4</sup>

The use of hyperbaric oxygen as adjunctive treatment for Zygomycosis has been reported since 1970s and hyperbaric oxygen treatment is usually well tolerated and is associated with low risk of adverse events.<sup>5</sup> Hyperbaric oxygen therapy (HBOT) has been used as an adjunct to aggressive surgical debridement, amphotericin B therapy, control of any underlying predisposing conditions by aiding neovascularization and subsequent healing.<sup>6</sup> Hyperbaric oxygen therapy has also been used to treat mucormycosis.<sup>3</sup> In the suspected case of mucormycosis at Naval Hospital dr. Ramelan Surabaya Indonesia, Hyperbaric oxygen therapy had a good outcome.<sup>7</sup>

Hsp 72 is a member of the Hsp-70 protein family that expresses *heat shock protein 70* kilodalton.<sup>8</sup> Heat shock protein 72 (HSP-72) is expressed in response to stress.<sup>9</sup> Heat shock protein 72 (HSP-72), a canonical intracellular molecular chaperone, may also function as an extracellular danger signal for the innate immune system.<sup>10</sup> Heat Shock Protein (HSP) 72 allegedly serves to protect the cell from stress. This protective role of Hsp-72 is known because of the effect of this protein on stress that activates the signal pathway of protein kinase.<sup>11</sup>

HSP was first identified in heat-exposed cells. Then it is known that many other stimuli that can result in the release of HSP in normal body temperature such as hypothermia, hypoxia or hyperoxia, decreased energy, acidosis, viral infection, ROS and RNS. HSP has 2 main functions: the physiological condition, acting as a chaperone that plays a role in protein folding and transport. Second, HSP triggers a cell response to stress, such as changes in temperature, presence of free radicals, bacterial and viral infections, heavy metals, ethanol and ischemia.<sup>12</sup> Tang *et al* (2007) demonstrated that proinflammatory signaling function of HSP-72 in macrophages.<sup>13</sup>

This study was conducted to analyze how the expression of Hsp-72 after therapy with hyperbaric oxygen in maxillary mucosal tissue of mucormycosis infection on tooth extraction.

## Materials and methods

Before this study was done, it has been preceded by two preliminary research processes in-vitro and in-vivo to observe the effect of hyperbaric oxygen on 10<sup>6</sup> *Rhizopus Oryzae* Strain CBS 110.17 (mucormycosis agent) and the healing effect of hyperbaric oxygen therapy on experimental animal (marmots) which made infected by mucormycosis on dental extraction.<sup>14</sup> This in-vitro study was to examine the effect of hyperbaric oxygen to the growth of mucormycosis agent *Rhizopus Oryzae* strain CBS 110.17 after fourteen times treatments with hyperbaric oxygen in macroscopic and microscopic aspects.<sup>15</sup> The result implicate that HBO have an effect on *Rhizopus oryzae* Strain CBS 110.17.

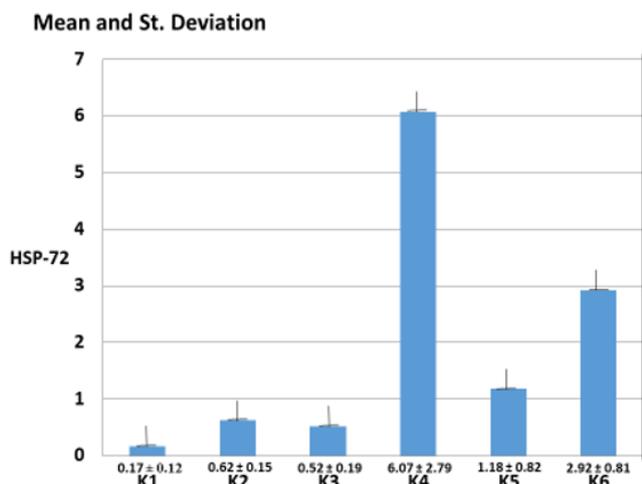
In the in-vivo preliminary study, it was found that hyperbaric oxygen therapy had an effect on repair of damaged gingival mucous tissue due to mucormycosis infection both in maxillary and mandibular after extraction. On the lower jaw (mandibular), with tooth extraction first and then fungal injection, compared with maxilla (fungal injection first and on the third day, the tooth extract), there was the differences in tissue damage. The tissue damage in maxillae got worse than the mandible, but after being given HBO therapy, the healing in the maxilla better than the mandible.<sup>16</sup>

In the study of the expression of Hsp-72 to the effect of Hyperbaric oxygen in mucormycosis infection in maxillary gingiva mucosa on dental extraction was a true laboratory experimental study with post test only control group design. Ethical permission of this research was obtained from the Ethics and Scientific Research Committee of Experimental Animal Use in Dentistry Faculty of Airlangga University. Thirty four (34) male marmots with mean age of 3-4 month and body weight 300-400 gram and were divided in 6 groups by random allocaton: 1 control group, 1 group of dental extraction and 4 intervention group. Two group, first with injection of 10<sup>6</sup>CFU/ml *Rhizopus oryzae* strain CBS 110.17 0,3 ml, after the third day, tooth extraction. The other two group, dental extraction first and then *Rhizopus oryzae* injection. The four intervention group were divided with none HBO and HBO treatment of 2,4 ATA 3x30 minute, one session, for 5 days. The expression of HSP-72 was examined by immunochemistry.

## Results

Variabel	Normal (n=6) K-1	Dental extraction (n=5) K-2	Rhizopus Injection→ Dental extraction Non HBO (n=6) K-3	Rhizopus Injection→ Dental extraction + HBO (n=6) K-4	Dental extraction→ Rhizopus Injection Non HBO (n=6) K-5	Dental extraction→ Rhizopus Injection +HBO (n=5) K=6
Hsp-72	0,17±0,12	0,62±0,15	0,52±0,19	6,07±2,79	1,18±0,82	2,92±0,84

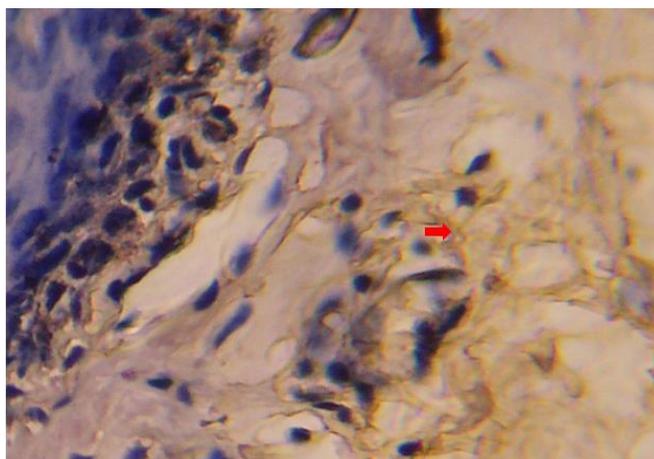
**Table 1.** Mean and Standard Deviation of Hsp-72 Expression in all Group.



**Figure 1.** Diagram of mean and standard deviation of Hsp-72 expression on macrophage in all group.

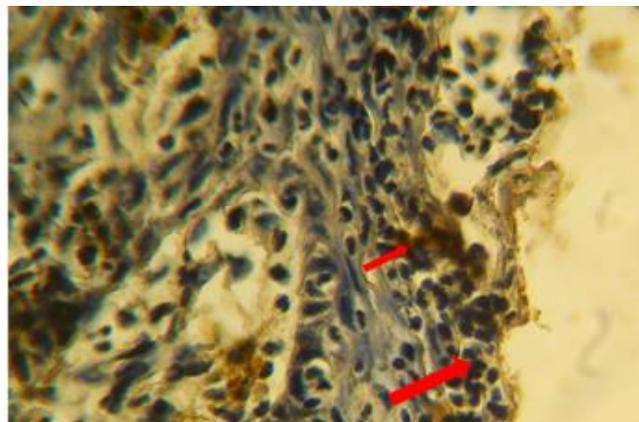
Variabel	K1 - K2	K1 - K3	K1 - K4	K1 - K5	K1 - K6
Hsp-72	3,6*	3 *	35,7*	6,9*	17,2*

**Table 2.** The comparative results of Hsp-72 expression between K1 and the other group.



**Figure 2.** Microscopic images of expression results of HSP-72 in Group-.3 : the action of tooth removal and fungal injection without HBO therapy.

A brown macrophage cell appears ( → ) to give a positive reaction to the anti HSP-72 monoclonal antibody on the periodontal tissue: Magnification 400 times.



**Figure 3.** Microscopic images of HSP-72 expression results in Group-6: the action of tooth removal and fungal injections with HBO therapy.

A brown macrophage cell appears ( → ) to give a positive reaction to the anti HSP-72 monoclonal antibody on the periodontal tissue: Magnification 400 times.

The above research data, in table 1,2 and also figure 1,2,3, all distributed normally with statistical test Kolmogorov-Smirnov Z one sample, then done parametric statistical test that is analysis on correlation, significance test and double comparison test between groups as in the next table 3 below.

No	Variabel	Normal (n=6) K1	Dental extraction (n=5) K2	Rhizopus Injection→ Dental extraction Non HBO (n=6) K3	Rhizopus Injection→ Dental extraction + HBO (n=6) K4	Dental extraction→ Rhizopus Injection Non HBO (n=6) K5	Dental extraction→ Rhizopus Injection + HBO (n=5) K6
1	Hsp-72	0,96	0,92	0,97	0,95	0,74	0,95

**Table 3.** Result of normality test of research variable in each group.

Variabel	Normal (K1)	Dental extraction (K2)	Rhizopus Injection→ Dental extraction -OHB (K3)	Rhizopus Injection→ Dental extraction + HBO (K4)	p Value
Hsp-72	0,17±0,12 <sup>a</sup>	0,62±0,15 <sup>b</sup>	0,52±0,19 <sup>b</sup>	6,07±2,79 <sup>c</sup>	<0,0001

**Table 4.** Comparative Test Results in Groups K1, K2, K3, K4

Wilks' Lambda ; F = 24,278 ; value p < 0,0001

**Note :** different superscript letters show significant differences (p<0,05) using a double comparison test

The table above shows the results of normality tests that have a value of p ≥ 0,05 , so

it can be said that the variables in each group is normally distributed. Table 4 and 5 below showed comparative test results between group.

Variabel	Normal (K1)	Dental extraction (K2)	Dental extraction → Rhizopus Injection -OHB (K5)	Dental extraction → Rhizopus Injection +OHB (K6)	p Value
Hsp-72	0,17±0,12 <sup>a</sup>	0,62±0,15 <sup>b</sup>	1,18±0,82 <sup>b</sup>	2,92±0,84 <sup>c</sup>	<0,0001

**Tabel 5.** Comparative Test Results in Groups K1, K2, K5, K6.

Wilks' Lambda ; F = 24,278 ; value p < 0,0001

**Note :** different superscript letters show significant differences (p<0,05) using a double comparison test.

## Discussion

Invasive fungal infections (mycoses) are uncommon, opportunistic, when organisms to which we are frequently exposed gain entry to the body due to a reduction in the host defenses, or through an invasive portal, such as a dental extraction.<sup>17</sup> Mucormycosis infection is an invasive, acute and rapidly progressing fungal infection with high mortality rate. Oral manifestations of these infection some time appeared as palatal ulcer and be triggered by tooth extraction and damage the mucosa gingiva and alveolar bone and it becomes necrotic.<sup>18</sup> A condition in which necrotic cell death clearly contributed to the release of Hsp-72 from cell is after severe trauma.<sup>19</sup> The aim of this study is to analyze the expression of Hsp-72 in maxillary mucosal tissue of mucormycosis infection on dental extraction after HBO therapy. From the results of the above study, the exposed expression of HSP-72 increased after the HBO therapy was given. In detail from table-1 and table-2, the following results show that the expression of HSP-72 on K-4 increases highest among other groups with a value of 6,07±2,79 (35,7 times to K-1 /normal), followed by K-6 with 2,92±0,84 ( 17,2 times to normal/ half of K-4). Group of tooth extraction, injection of the fungus without HBO (K-5) which is 1,18±0,82 ( 6,9 times to normal) and K-2 ( tooth extraction) with 0,62±0,153(3,6 times to normal) and the smallest is K-3 ( fungus injection, tooth removal without HBO ) only 0,52±0,19 ( 3 times to normal ). The results of comparative test in groups K1-K2-K3-K4 in table 4 and K1-K2-K5-K6 in table 5 show a significant difference for treatment with HBO and non HBO.

According to the theory that HSP-72 always increases when there is trauma to the

tissues that trigger the onset of immunity from the body, it is also mentioned by Tang D. *et al* (2007) which is said to represent the universal response of various stresses, cells will rapidly express and induce HSP among others HSP-70, as a major factor of inducible protein. Tang *et al* demonstrated that anti-inflammatory effect of HSP-72 is involved in inhibition of HMGB1 release and its proinflammatory signaling function in macrophages.<sup>13</sup> Intra-cellular HSP-72 as a companion molecule to maintain homeostatis and cell survival. Under psychological conditions, HSP-72 predominantly localized in the cytosol. In response to inflammation and oxidative stimuli, HSP-72 can translocate into the nucleus which will provide a protective role against the stress around it. Proof of the accumulation of support of the HSP-72 translocation that is of significance satisfies rapid and immunoregulatory protection. For example, HSP-72 translocation in the nucleus is essential for apoptosis-suppressing activity.<sup>13</sup> HSP-72 linkage with HBO, Vince RV *et al* (2011) examined the effects of HBO pre-condition on HSP-72 expression for in-vitro stress of human monocytes cells. He reported that the benefits of HBO pre-conditions were not to induce the exposure of HSP-72 in the circulatory blood cells but maybe associated with an increase in the response due to stress.<sup>20</sup> Taylor L. *et al* (2012) studied the effects of a hyperbaric environment on HSP-72 expression in vivo. From this study it is said that HSP-72 expression is disturbed after exposure to high pressure air when compared with the control of hyperbaric oxygen.<sup>9</sup> Williams JHH and Ireland HE (2008) state that the HSP-72 release is increased at 1 to 3 hours after stimulation , as well as secretion by cells that are apoptotic and released from necrotic cells. HSP-72 is also very potential as a hazard signaling together with HMGB-1 which plays a role in intracellular but strongly interacts with the immune system when found in extracellular. Hsp-72 protects cells from stressor through roles in intracellular.<sup>21</sup> One potential tool for treating invasive fungal infections is hyperbaric oxygen.<sup>22</sup> The use of hyperbaric oxygen as adjunctive treatment for Zygomycosis has been reported since 1970's and hyperbaric oxygen treatment is usually well tolerated and is associated with low risk of adverse events.<sup>5</sup> Gopalakrishnan (2012) said that hyperbaric oxygen therapy is theoretically attractive because it reverses the

ischemic acidotic conditions that perpetuate fungal growth. Hyperbaric oxygen treatments are usually given at two atmospheres for 1 hour on a daily basis for up to 30 treatments.<sup>23</sup> This may limit deformity by decreasing the required area of debridement. The raised oxygen pressure achieved with HBO treatment seem to improve the capacity of neutrophils to kill organism.<sup>24</sup> In addition, by reversing lactic acidosis, treatment with HBO complement the oxidative action of Amphotericin B. HBO therapy for mucormycosis should comprise of exposure to 100% oxygen, each dive ranging from 90 minutes to 120 minutes at pressures from 2.0 to 2.5 atmospheres with 1 or 2 exposures on a daily basis for total of 40 treatments. Information on treatment of mucormycosis with HBO is scarce and its role is in doubt.<sup>25</sup>

This study showed that the highest increase is K-4 with value  $6,07 \pm 2,79$  (35,7 to normal in Table-2), injected with  $10^6$  *Rhizopus oryzae* strain CBS 110.17 in 0,3 cc and tooth extraction on day 3 and given HBO therapy for 5 days. Whereas in K-6, extract the teeth first and injection of *Rhizopus oryzae* and given HBO therapy, only increased 17,2 to normal ( $2,92 \pm 0,84$ ). If compared between K-2, K-3 and K-5 with K-1 (normal) in table-2, it is found that the lowest value of HSP-72 is at K-3 (3) then K-2 (3,6) and K-5 (6,9). The decreased expression of HSP-72 in K-3 compared to K-2 is seen when the mucormycosis agent ( $10^6$  *Rhizopus oryzae* strain CBS 110.17) entering the tissues. In the expression of HSP-72 after tooth extraction (K-2), there is an increase, but in K-3 actually decrease. So, it is predicted that the trauma due to injection of *Rhizopus oryzae* strain CBS 110.17 more severe than tooth extraction (K-2) which causing HSP-72 decrease because HSP-72 is no longer able to protect the tissue. Whereas in K-5, tooth extraction first and then given the injection of *Rhizopus oryzae* strain CBS 110.17, even seen there is an increase more than 2 times from K-3 and K-2.

With this action, HSP-72 expression more increase at K-5. The result show that there is difference of HSP-72 expression in K-3 and K-5 and this can indicate that the condition in K-3 more severe than in K-5 which HSP-72 still react while in K-3 even decreased. By looking at the results of research and the opinions of experts, it is clear that the expression of HSP will always increase when there are disorders such as stress,

trauma or inflammation that attacks the human body to provide cellular protection.<sup>8,11,26</sup> The presence of tissue necrosis of the gingival mucosa in K-3, K-4, K-5 and K-6. In the group receiving HBO (K-4 and K-6), Hsp-72 expression remained highest in K-4.

This suggests that HBO therapy increases the expression of HSP-72 in necrotic tissue where mucormycosis can cause tissue necrosis.<sup>6</sup> In shock and stress stimuli, HSP-72 will be synthesized in intracellular where it will protect cells from protein denaturation or from death.<sup>11</sup> In the group receiving HBO (K-4 and K-6), although receiving different trauma, HSP-72 expression still increased sharply especially at K-4 (injected *Rhizopus oryzae* first) with the highest value. In K-6 (tooth extraction first) due to exposure from HBO, the value of the HSP-72 expression is half of K-4. This means that the trauma cause by *Rhizopus oryzae* injection (mucormycosis agent) is greater than the trauma of tooth extraction. Fungal infections that already exist in the oral cavity and then had tooth extraction will lead to more severe conditions than tooth extraction first and then exposed to fungal infection. Oter S, *et al* (2005) said that HBO is therapeutic modality with dual effect: it produces oxidative stress by itself but reduces oxidative stress when used in pathologic conditions.<sup>27</sup> Specifically about the expression of HSP-72 associated with HBO therapy as found by Vince RV *et al* (2011), did not induce HSP-72 expression, but it would be different after first having trauma and stress as in this study.<sup>20</sup> These animals get an injection treatment of  $10^6$  *Rhizopus oryzae* strain CBS 110.17 and tooth extraction or vice versa and were given HBO therapy will produce a drastic increase of HSP-72 expression.

## Conclusions

Hyperbaric oxygen therapy 2,4 ATA 3x 30 minute, one session during 5 days, increased the expression of Hsp-72 very significantly in mucormycosis infection (*Rhizopus oryzae* species strain CBS 110.17) on dental extraction.

In the maxillary mucosa that previously had mucormycosis (*Rhizopus oryzae* species strain CBS 110.17) injection and then performed tooth extraction was more pathogenic and progressive when compared with uninfected mucormycosis which is marked by decreased protection in

macrophage. In the oral mucosa that was previously not infected with mucormycosis, the role of HSP-72 still exists.

### Declarations of interest

The author report no conflict of interest for this article

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