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Administration of Vaccine Preservative Thimerosal Produces Impairment in Rat Liver

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HIGHLIGHTS

- Toxic effects of thimerosal on rat liver.
- Thimerosal increased the the level of ALT, ALP and AST.
- Activity of CAT, SOD, POD GSH and protein was reduced.
- DNA damage was observed.

Abstract: The present research was planned to analyze the toxic effects of thimerosal on rat liver. Mercury and mercury compounds are universally known toxicants for animals and humans. Thimerosal is widely used in the vaccines as a preservative which contains 49.6% mercury. Twenty-four adult male albino rats were distributed into four groups (n=6). The first group was considered as a control group. While, second, third and fourth groups were intoxicated with 0.5, 10 and 50 µg/kg thimerosal (i.m.) respectively. After 30 days, rats were slaughtered to analyze the liver tissues. The results of the experiment exposed that thimerosal

instigated significant ($p<0.05$) increase in alanine transaminase (ALT), alkaline phosphatase (ALP) and aminotransferase (AST) levels. Catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) activities and *Glutathione (*GSH) and protein levels were significantly ($p<0.05$) reduced. Furthermore, significant increases in Hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substances (TBARS) level and DNA damage was observed. Histopathological study revealed severe damages, e.g. fatty alterations, deterioration of lobular structure and degeneration of nuclei in hepatic tissues of thimerosal treated rats. Results of present investigation revealed that thimerosal induces hepatotoxicity at different levels.

Keywords: thimerosal; reactive Oxygen species; oxidative stress; hepatotoxicity.

INTRODUCTION

Toxic effects of mercurial compounds have been observed in humans and animals. These compounds damage the central nervous system (CNS), kidneys, immune system and cardiovascular system [1]. Generally, inorganic forms of mercury are associated with renal damage and organic form of mercury induces damage in the nervous system [2].

Thimerosal (sodium ethyl mercury thiosalicylate) contains 49.6% of mercury (Hg) of its total weight. The use of thimerosal was approved in 1976 by “Drug and Food Administration of United States” and it is being extensively used in vaccines as preservative till the to-date [3]. Thiomersal is also used as a wound disinfectant and preservative agent in medicines [4], and in preparations of different biological products [5]. In 2009, thimerosal was used mostly in anti-flu vaccines to counter the swine influenza [6].

Thimerosal containing vaccines are the central path of mercury exposure in the hospitals [7]. Mercury affects the nervous system of infants and causes several disorders such as speech delay, autism, confusion, learning disabilities and attention deficit hyperactive disorder in them [8]. Thimerosal causes autism spectrum disorder (ASD) and neurodevelopmental disorders [9] High dose of thimerosal containing products has toxic effects [10]. Several studies have also shown impaired binding of secondary messengers and dysregulation of neurotrophin signaling followed by thimerosal exposure [11].

Thimerosal induced damage impacts on epithelial tissues, e.g. contact and conjunctivitis dermatitis have been formerly analyzed [12]. In aqueous salty solution, thiomersal rapidly breaks into ethyl mercury chloride and ethyl mercury hydroxide [13]. Thimerosal can produce the ROS in mammalian cells [14]. Till the date, toxic effects of thimerosal have not been investigated. Therefore, current research was planned to assess the toxic effects of thimerosal on the rat liver.

MATERIAL AND METHODS

Experimental design

Thimerosal was purchased from Sigma-Aldrich (Germany). Rats were placed in animal house of the Department of Pharmacology. Twenty-four male rats (*Rattus norvegicus*), weighing (150-180 g) were selected. Rats were separated into four groups having equal number of rats (six) in each. “Group 1 named as a control group, received 0.9% of saline through oral gavage. Rats of the group 2” were treated with 0.5µg/kg thimerosal through intramuscular injection (i.m.). Group 3: Rats of this group were administrated with thimerosal 10µg/kg (i.m.**)**. Group 4: Rats of this group received a dose of 50µg/kg thimerosal (i.m.). Duration of the treatment was thirty days.

Ethical approval

This experiment was conducted after the approval of ethical committee of the University of Agriculture, Faisalabad (D. No. 2836/*ORIC*).

Sample collection

All rats were anesthetized with diethyl ether and dissected on the 31st day of the trial and blood was collected in EDTA tubes. Centrifugation of blood was carried out for 15 min. at 3000 rpm for plasma separation and stored at -20 ^{o}C. Liver of each rat was detached, weighed, washed with ice cold saline and processed for further analysis. Liver parts were also preserved in 10% formalin solution in glass vials for histopathology and stored in zipper bags at -20 ^{o}C for antioxidant enzyme analysis. Superfluous rats residual was buried by qualified laboratory technician according to the guidelines of U.S. bio-security agencies.

Determination of liver function markers

Levels of functioning markers of liver like AST, ALT and ALP were assessed by means of relevant commercially available abcam diagnostic kits.

Estimation of antioxidant enzyme activity

CAT and POD activity were evaluated through the procedure of Chance and Maehly [15] after few amendments. Procedure of Kakkar and coauthors [16] was followed to measure the SOD activity. The level of GSH was measured with the spectrophotometric protocol [17].

Estimation of total protein content

Procedure of Lowry and coauthors [18]. was tailed for the assessment of total protein content in liver tissues.

Estimation of TBARS and H_2O_2 level

TBARS level was evaluated by comprehending the procedure of Iqbal [19]. The Hydrogen peroxide (H_2O_2) level was assessed by following the procedure of Pick and Keisari [20].

Comet Assay:

DNA damage was assessed by the methodology of Dhawan [21] through neutral comet assay. Pasteurized “slides were immersed in 1% NMA (Normal melting agarose) and let it set at normal room temperature”. In 1 mL of cold lysis solution, a minor section of liver tissue was placed and crushed in slight fragments and then added 75µl LMA (low melting agarose; 1%). This prepared solution was spread smoothly over formerly coated slides. These slides were placed in the ice box for almost 8 to10 min., then the cover slips were amputated and slides were placed again in the ice box and set these to solidify. After that, third coating of LMA was processed and slides were over placed in the lysis mixture for approximately 10 minutes then rested in freezer for two hours. After that staining of slides with ethidium bromide (1%) was carried out and studied under a fluorescence microscope. Image software (TRITEK) was employed for the assessment of DNA injury level. Parameters such as comet number, tail moment, head length, comet length, olive moment, tail length, % DNA content in the tail and head were considered for DNA integrity.

Histopathological assessment

For histopathological assessment, liver tissues were collected and washed with cold normal saline and fixed in neutral buffered formalin solution (10%) for histopathological examination. Tissues were dehydrated in ascending grades of alcohol (80%,90% and 100%) and embedded in paraffin wax. For the preparations of slides, thin slices of embedded hepatic tissues (almost 4-5 µm) were cut by using rotary microtome, stretched on slides and stained with hematoxylin/eosin stain. Light microscope (Nikon, 187842, Japan) was used for histopathological study at 40x magnification.

Statistical analysis

Data was shown as Mean ± SEM. For the comparison of different groups one-way ANOVA followed by Dunnett’s test was applied by using Minitab software. Level of significance was fixed at $p<0.05$.

RESULTS

Effect of thimerosal on liver function markers:

Thimerosal administration significantly ($p<0.05$) increased the hepatic function markers AST, ALP and ALT in treated rats in comparison to the rats of the control group”. Thimerosal increased the levels of liver function markers in a dose dependent manner (Table 1).

Table 1. Effect of thimerosal on protein level and liver function markers ALT, AST and ALP in rats

Treatments	ALT (U/L)	ALP (U/L)	AST (U/L)
Control	58.67 ± 3.28 [a]	65.00 ± 2.35 [a]	49.33 ± 2.90 [a]
Thimerosal (0.5 µg/kg)	85.33 ± 4.25 [b]	92.33 ± 2.37 [b]	75.33 ± 3.17 [b]
Thimerosal (10 µg/kg)	93.33 ± 4.25 [b]	94.00 ± 2.16 [b]	84.67 ± 2.60 [c]
Thimerosal (50 µg/kg)	188.7 ± 9.49 [c]	113.3 ± 1.96 [c]	158.3 ± 4.40 [d]

Means that do not share similar letters are significantly different.

Effects of thimerosal on antioxidant enzyme activity and total protein content:

Thimerosal administration disturbed the antioxidant enzyme activity. CAT, POD, SOD activities and GSH level were significantly ($p<0.05$) reduced in the thimerosal administrated groups when compared with the control group. Thimerosal decreased the activities and level of these anti-oxidant enzymes in a dose dependent manner. Moreover, the protein content in liver tissues significantly ($p<0.05$) reduced in a dose dependent means in all the treated groups compared to the control group (Table 2).

Table 2. Effect of thimerosal on antioxidant enzymes CAT, POD, SOD, GSH and total protein content in hepatic tissues

Treatments	CAT (U/mg protein)	POD (U/mg protein)	SOD (U/mg protein)	GSH (µM/g tissue)	Protein content (mg/g)
Control	7.53 ± 0.11 [a]	4.43 ± 0.11 [a]	8.43 ± 0.12 [a]	16.62 ± 0.19 [a]	5.02 ± 0.05 [a]
Thimerosal (0.5 µg/kg)	6.23 ± 0.07 [b]	3.85 ± 0.03 [b]	5.87 ± 0.04 [b]	14.80 ± 0.09 [b]	4.39 ± 0.02 [b]
Thimerosal (10 µg/kg)	6.07 ± 0.04 [b]	3.71 ± 0.02 [b]	3.65 ± 0.03 [c]	14.07 ± 0.07 [b]	4.27 ± 0.03 [b]
Thimerosal (50 µg/kg)	4.55 ± 0.17 [c]	3.03 ± 0.03 [b]	2.78 ± 0.01 [d]	12.02 ± 0.05 [c]	3.97 ± 0.04 [c]

Means that do not share similar letters are significantly different

Impacts of thimerosal on H_2O_2 and TBARS:

H_2O_2 and TBARS level were significantly ($p<0.05$) increased in thimerosal administrated “rats in comparison to control. Thimerosal increased the levels of TBARS and H_2O_2 in a dose dependent manner” (Table 3).

Table 3. Effects of thimerosal on TBARS and H_2O_2 level in hepatic tissues

Treatments	H_2O_2 (nM/min/mg protein)	TBARS (nm TBARS/min/mg tissue)
Control	1.69 ± 0.04 [a]	15.57 ± 0.61 [a]
Thimerosal (0.5 µg/kg)	2.19 ± 0.06 [b]	17.84 ± 0.59 [b]
Thimerosal (10 µg/kg)	2.41 ± 0.01 [b]	18.14 ± 0.26 [b]
Thimerosal (50 µg/kg)	2.92 ± 0.02 [c]	21.91 ± 0.14 [c]

Means that do not share similar letters are significantly different.

Effect of thimerosal on comet parameters:

Current findings indicated that thimerosal produced the DNA damages in liver cells. Tail length, comet length, olive moment, tail moment and % DNA in the tail remarkably ($p<0.05$) enhanced in comparison to control. Moreover, % DNA in head and head length were substantially ($p< 0.05$) reduced in thimerosal treated groups while compared to control (Table 4, Figure 2).

Table 4. Effects of thimerosal on comet parameters of hepatic cells in rat.

Groups	Control	Thimerosal (0.5 µg/kg)	Thimerosal (10 µg/kg)	Thimerosal (50 µg/kg)
No. of comets	28.66 ± 1.20 [a]	35.13 ± 1.16 [b]	39.85 ± 0.85 [c]	50.77 ± 1.71 [d]
Comet length	40.2 ± 1.14 [a]	47.4 ± 0.83 [b]	52.14 ± 1.02 [c]	60.72 ± 1.15 [d]
Tail length	5.98 ± 0.07 [a]	6.33 ± 0.01 [b]	6.95 ± 0.03 [b]	07.32 ± 0.05 [c]
Head length	38.22 ± 0.64 [a]	30.22 ± 0.54 [b]	29.62 ± 0.43 [b]	24.44 ± 0.27 [c]
% in tail	6.02 ± 0.08 [a]	10.11 ± 0.12 [b]	13.32 ± 0.54 [c]	20.55 ± 0.81 [d]
% in head	93.97 ± 0.08 [a]	89.88 ± 0.12 [b]	86.01 ± 0.13 [b]	79.45 ± 0.81 [c]
Olive moment	2.18 ± 0.04 [a]	2.68 ± 0.01 [a]	3.04 ± 0.03 [b]	3.24 ± 0.03 [b]
Tail moment	0.69 ± 0.00 [a]	1.21 ± 0.01 [b]	1.35 ± 0.01 [b]	1.95 ± 0.03 [c]

Means that do not share similar letters are significantly different.

Effect of thimerosal on liver histology

Thimerosal induced morphological damages in rat liver are displayed in Figure 1. Rats of control group exhibited standard structural patterns of liver with normal sinusoids and central veins as demonstrated in Figure 1A. But Thimerosal intoxication resulted in the serious liver impairments such as a remarkable increase in the fat deposits, degenerated structure of the lobules and infiltration of inflammatory cells with dilated sinusoids in a dose dependent manner as shown in Figures 1 B, C, D.

Thimerosal, a mercury derived compound, is made up of ethyl mercury and thiosalicylic acid. It is used in vaccines as a preservative [22]. Liver confronts the toxicants that reach in the liver, portal vein through the digestive system. Liver functions can be disturbed due to injury caused by severe or long-lasting exposure to toxicants [23]. The present study was conducted to assess biochemical, histopathological and DNA damage in liver due to thimerosal.

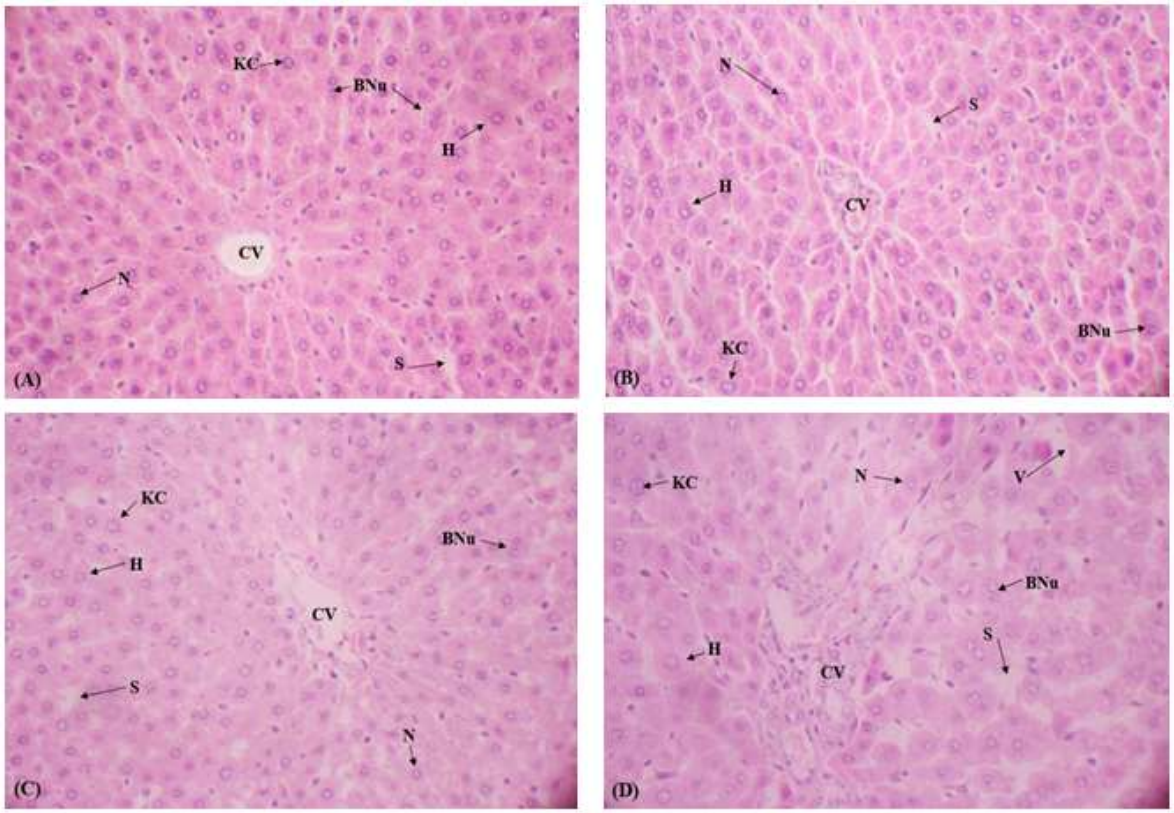

Figure 1. Effects of thimerosal on liver histology (40x, H&E). **A)** Control group. **B)** Thimerosal (0.5 µg/kg). **C)** Thimerosal (10 µg/kg). **D)** Thimerosal (50 µg/kg). CV; Central venule, S; Sinusoids, H, Hepatocytes; KC, Kupffer cells; N, Nucleus; CNe, Centrilobular necrosis; BNu, binucleated; V, vacuoles.

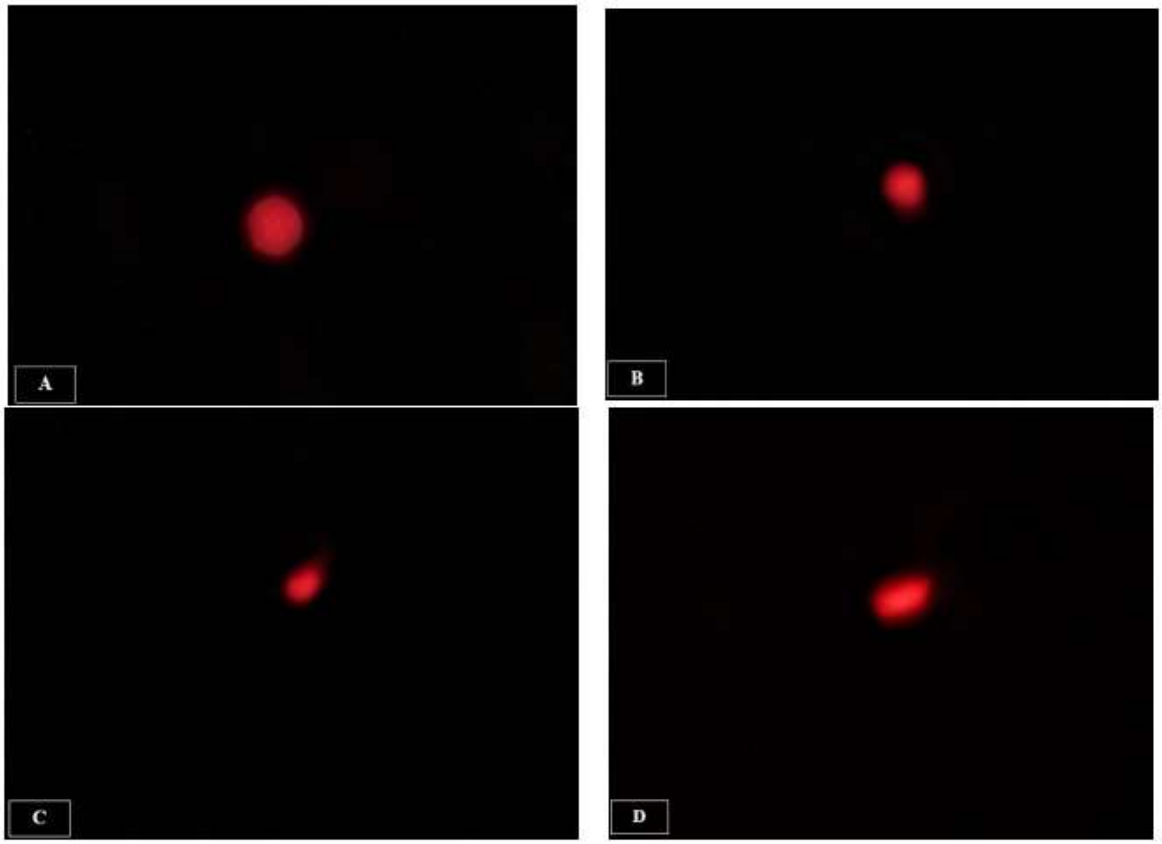

Figure 2. Fluorescence photomicrograph of liver cells and genotoxic outcome of thimerosal (**A**) Control (**B**) Thimerosal (0.5 µg/kg) treated (**C**) Thimerosal (10 µg/kg) treated (**D**) Thimerosal (50 µg/kg) treated.

DISCUSSION

AST, ALT and ALP represents the measurable liver function markers. In current research, a remarkable increase in serum biomarkers was observed in thimerosal treated rats, hereafter evincing the fact that due to structural damage of hepatocytes, enzymes were discharged into the bloodstream instead of residing in the cytoplasm. The unusual increase of these enzymes in blood is always associated with hepato-necrosis [24]. This can be evidently observed in the histopathology of thimerosal treated groups (Figures 1 b, c, d) in which cellular infiltration, degeneration and colossal necrosis of hepatocytes happened afterwards several cytosolic marker enzymes of liver were moved into the blood stream [25]. The significant increases in these markers confirmed that liver performance was disturbed, which was further confirmed by acute histopathological alterations.

CAT, SOD and POD (antioxidant enzymes) are essential regulators of oxidative stress in the cell. SOD changes superoxide to H_2O_2 and participates as an initial defense line against its lethal impacts [26]. CAT deteriorates H_2O_2 into water and oxygen and protects the tissues from damage due to lipid peroxidation in the biological system. In this research, activities of anti-oxidant enzymes like CAT, POD, SOD and level of GSH in liver tissues were remarkably reduced. Reduced activities of antioxidant enzymes, level of GSH and protein in serum showed that the acute liver damages were induced due to thimerosal. It seems that the administration of thimerosal causes oxidative stress in the liver via the production of free radicals. According to Sharpe and coauthors [27], decrease in the antioxidant enzyme levels leads to oxidative stress and thus cell becomes unable to detoxify ROS produced within the cell. The overwhelming production of free radicals in turn further suppresses the activities of antioxidant enzymes. In this experiment, decrease in total protein content showed that the injuries were due to mercury containing compounds [28].

Thimerosal increased H_2O_2 formation, accompanied by amplified peroxidation of mitochondrial lipids, which leads to oxidative stress. Hence, mercury containing compound are known to elevate hydrogen peroxide due to increase of ROS. Thimerosal increased the level of the TBARS. Thimerosal exposure produced free radicals that caused damage in lipids, proteins and DNA in the body. Hepatic tissues are extremely susceptible to the toxic chemicals which results in hepatic cirrhosis which leads to death. Peroxidation of the polyunsaturated fatty acids may lead to increased levels of TBARS which is the final

product of lipid peroxidation and considered as oxidative stress biomarker [29]. TBARS are involved in the destruction of the membrane system of the cell and fusion of extremely reactive H_2O_2 which increases its capability to react with proteins and DNA.

ROS production in the cells can instigate deterioration in cell membrane and DNA damage that can be authenticated by examining histopathology and comet assay. Comet assay has turned out to be a basic technique to measure DNA impairment [30]. DNA is affected by several toxins whose commotion escorts to genotoxic consequences [31] and breakdown of double or single strand might also occur in DNA by disturbing its unified form. In the present research, comet assay was operated for the evaluation of damage in various DNA parameters together with tail length, head length, comet length, % DNA in the head and %DNA in tail moment. The rise in the tail length, tail moment, comet length and % DNA in tail samples of liver cells were observed when treated with thimerosal. Thimerosal raised the constrictions replicate DNA breakdowns and destructions of the membrane system of liver cells. Corresponding toxicity enhanced the liver damages and functional irregularities, though % DNA in head remained reduced within thimerosal treated rats [32]. Thimerosal caused DNA damage in liver cells is conscientious for DNA relocation from comet head to tail, consequently escalating the % DNA in tail, tail moment as well as tail length as reported in Ali and coauthors [33] against standard toxin.

In the current study, blockage of hepatoportal blood vessels, blockage of central vein and fatty alterations in portal tract of treated rats demonstrated the toxic effects of thimerosal compound. Damage in liver histology was in line with elevation in hepatic marker enzyme which shows cellular damage of hepatocytes [34]. These damages in liver tissues are may be due to lipid peroxidation, which caused tissue degeneration and deterioration of cell membranes as observed in standard toxin intoxication [33].

CONCLUSION

The consequences of this study exhibited that thimerosal at its low (0.5 µg/kg), medium (10 µg/kg) and higher (50 µg/kg) doses induced oxidative stress and disturbed the liver in a dose dependent manner and highest damage was observed at 50 µg/kg. The results of the present research propose novel discernments about possible hazards of thimerosal exposure to animals and might additionally impart basic data regarding to the potential hazardous effects of thimerosal on human health. Thus, the use of thimerosal as animal and human vaccine preservative should be of great concern, specifically tell the efficient risk evaluation. More studies are required to investigate the molecular basis of these changes both in vivo and in vitro, which will help to identify that how thimerosal influences the physiology of various tissues within the body.

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