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# The Effect of Silver Nanoparticles on BRAF Gene Expression

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## ABSTRACT

The recent studies suggested the possible toxicities or genetic alterations associated with biological and medical applications of silver nanoparticles (AgNPs). The current research is directed to see if AgNPs administration can lead to some changes in expression of BRAF gene in selected body organs tissues. Fifty-six male of *musmusculs* (Balb/C) mice from the animal house of Al-Nahrain Centre of Biotechnology were used. These animals were divided randomly to seven groups (eight mouse in each group), one of these groups represented the control group, three groups were subjected to different doses of AgNPs (0.25, 0.5and 1 mg/kg of body weight) for one week, and the remaining three groups were subjected to three different doses of AgNPs (0.25, 0.5and 1 mg/kg of body weight) for two weeks. Liver, spleen, brain and kidneys tissue samples from each mouse in all groups (including control group) were collected, RNA was extracted and cDNAs were synthesized and then used for real time PCR and qRT-PCR Analysis. The data generated in this study indicates that the hepatic and spleen tissues expression of BRAF gene is significantly linked to AgNPs administration, also, these data showed a high significant relation between AgNPs administration and the expression of this gene in brain and kidneys tissues.

**Keywords:** AgNPs; BRAF; Al-Rafidain University College.

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## INTRODUCTION

The last decades shows an increment in the production of different types of material containing synthesized nanoparticles (NPs), because their synthesis is became easier, in addition to the growing need of these NPs to be involved in different industrial fields (1, 2). Silver nanoparticles (AgNPs) represent the most important kind of NPs for many reasons, like antibacterial activity against various species (3, 4, 5), affordable manufacturing cost (6), and their ability to be synthesized in different shapes (7). AgNPs are involved in wide range of daily used medical and healthcare products, furthermore, they are used in the food, sports and clothing industries (8; 9; 10). The released Ag+ ions from AgNPs are extremely worrying as they can cause toxicity (11). The safety of AgNPs is an important point to be considered before estimating their effects (12). Despite some studies considered the release of Ag+ ions are the main cause of AgNPs possible toxicity (13; 14; 15), but other studies said that the toxicity of these nanoparticles might not be related to Ag+ ions dissolution only (16; 17). However, some studies reported recently that in some cases, metal nanoparticles can be more toxic than the metal ions their selves (18; 19).
The BRAF protein which encoded by a BRAF gene is involved in one of the signaling pathway (MAP kinase/ERK), which regulates some important cell functions (20). The products of BRAF gene have been studied, and their actions reported to be contradictory as some BRAF gene products were act as activator to the RAS/MAPK pathway, while the other products were act as silencer to this pathway (21; 22; 23; 24; 25). Furthermore, it had been found that overexpression of this gene or its hyperactivity can interfere with the RAS/MAPK pathway, which can result in many serious developmental disorders (26,27,28), and also it can lead to various cancer types (29). Also, somatic mutations of this gene have been approved in Langerhans cell histiocytosis (30; 31), Erdheim– Chester disease (32), and may types of human cancers (23; 33; 34; 35; 36; 37).
The current study was conducted to examine the effects of silver nanoparticles (AgNPs) on of BRAF gene expression in selected body organs tissues (Liver, Spleen, Brain and Kidneys).

## MATERIAL AND METHODS

### Synthesis of Silver Nanoparticles

The AgNPs were prepared and characterized in previous study by Nasir *et al.*, (2016), who synthesized these particles as (40±5) nm in size and like spherical in shape and used olive leaves extract as a reducing agent and D-sorbitol as capping agent (38).

### Experimental Animals

In this experiment, fifty-six male of *musmusculs* (Balb/C) mice (weighted 23-35 g, ages 8-10 weeks) from the animal house of Al-Nahrain Centre of Biotechnology were used. The mice were kept in plastic cages under controlled conditions of temperature (23-25) °C, and a 12-h light / dark cycle (39), and left for 7 days for acclimation period before starting the experiment (40). The animals were divided randomly to seven groups, each group contained eight mice in independent cage, and the mice were fed a standard pellets diet with enough water daily as a follow:

- The 1st and 4th groups were challenged intraperitoneally dose of 0.25mg/kg of body weight of AgNPs (50 µl) for 7 and 14 consecutive days respectively.

- The 2nd and 5thgroups were received 0.5mg/kg of body weight of AgNPs (50 µl) intraperitoneally for 7 and 14 consecutive days respectively.
- The 3rdand 6thgroups were challenged intraperitoneally dose of 1mg/kg of body weight of AgNPs (50 µl) for 7 and 14 consecutive days respectively.
- The 7th group was left as a control group without receiving AgNPs solution with intraperitoneally dose (50 µl) of distilled water.

**Tissue Sampling**

At the next day of the end of the dosing period (after 7 days), the 1st, 2nd and 3rd groups of mice were sacrificed by cervical dislocation (Euthanasia) (39), while the 4th,5thand 6thgroups of mice were sacrificed at the next day of the end of the dosing period (after 14 days). The mice of the 7th group (control group) were also sacrificed by cervical dislocation (Euthanasia).

**Tissue preparation for RNA extraction**

Tissues have been prepared for histopathological examination according to the method of Junqueira and Carneiro (2003), using the paraffin sections technique for livers, spleens, kidneys and brains (41). Tissue paraffin blocks were cut using electrical ultra-microtome to prepare 5-8 sections of semi-thin sections (0.5-10µm) from each block and placed in Eppendorf tube to extract RNA samples.

**Pre-Extraction Preparations**

**1**- DNase I Stock Solution was prepared by dissolving the lyophilized enzyme in 550 µl RNase-free water.

**2-** RPE Buffer was prepared by adding an amount of 44 µl of absolute ethanol (96-100%) to the bottle containing 11µl buffer RPE concentration.

Purification of total RNA from Formalin Fixed Paraffin Embedded (FFPE) tissue sections was made by using Qiagen Kit and RNA Extraction also done by using Qiagen Kit, the eluted RNA was placed in new tubes and stored at (-20°C).

**cDNA Synthesis (Reverse Transcription):**

The total RNA was reversed transcribed to complementary DNA (cDNA) using High Capacity RNA-to-cDNA kit, Applied Biosystem, Part No.4387406. The cDNAs were then stored at -20°C to be later used in real time PCR. RT-PCR Analysis.

**Table 1:** Thermal Cycling Condition Program

| **Temperature** | **Heated Lid** | **Step 1** | **Step2** | **Step3** | **Step4** | **Step5** |
|---|---|---|---|---|---|---|
| | 111ºC | 25ºC | 37ºC | 42ºC | 75ºC | 4ºC |
| Time(min.) | -- | 10 | 10 | 60 | 5 | ∞ |

**Note: These conditions are optimized for use with High-Capacity cDNA Reverse Transcription Kits.**

Quantifications were performed in duplicate for each sample using SYBR Green master mix. Duplicate reactions showing differences of more than 0.3Ct were repeated. Two non-template controls were also included in each run. Control gene mRNA concentrations were amplified and used to normalize the BRAF mRNA levels, and correct synthesis of cDNA as well as the calculations descriptions. Primers designing were done using Primer 3 Plus for BRAF gene and B-ACTIN gene as a control gene.

**Table 2:** The Primers used for BRAF and B-ACTIN genes.

| **Primer** | **Sequence (5' →3' direction)** | **Melting temperature Tm (ºC)** |
|---|---|---|
| B-ACTIN - F | CCTGAACCCTAAGGCCAAC | 60 |
| B-ACTIN - R | ACGTACATGGCTGGGGTGT | 62 |
| BRAF-F | GTCTCAGTTCAGGTGTCCTTCCTG | 63.9 |
| BRAF-R | AGACGTCTTGTGTCGTGGCTACTG | 65.9 |

The adopted method during qRT-PCR was the relative quantization (RQ), which uses the comparative cycle threshold (CT) to determine the change in the expression of the nucleic acid sequence (target) in a sample relative to the same sample at time zero (42).

SYBR Green assay was performed in a 20µl reaction arranged (10 µl of master mix SYBR green, 4.6 µl of water, 0.6 µl primer, 3 µl cDNA). Plate was setup in 96-well plate for each gene. The qRT-PCR protocol for SYBR Green assay was as a follow:

Stage 1: 50 ºC for 2min., Stage 2: 95ºC for 10 min., Stage 3 included a three- steps cycle procedure (denaturation 95ºC for 10 sec., annealing 62ºC for 15 sec. and extension 72ºC for 45 sec.) repeated for 50 cycles, then cooled to 40ºC for 10 sec.

**QRT-PCR Data Analysis (**42**)**

The gene expression levels, and fold change were quantified by measuring the threshold cycle (Ct), which is linked to the target molecules concentration in the reaction in an opposite manner. The Ct data were normalized by using endogenous control gene (B-ACTIN gene). For a selected control gene, the arithmetic or geometric mean of Ct values is considered the normalization factor.

**ΔCT sample = Ct sample-Ct endogenous control**

**ΔCT calibrator = Ct control- Ct endogenous control**

The normalized data of ΔCT are applied in the measurement of the relative fold change of gene expression, by using specific calibrators (control sample):

$$\Delta\Delta CT = \Delta CT\ sample - \Delta CT\ calibrator$$

$$\text{Relative copy number} = \text{Fold change} = 2^{-\Delta\Delta CT}$$

## RESULTS

**BRAF Gene Expression**

The results showed that there were variable tissue expression levels of BRAF gene in different organs (liver,

spleen, brain and kidneys) among experimental groups and also between experimental groups and control, according to the given dose of AgNPs and /or duration of AgNPs administration (Table-3).

**Table 3:** Effect of different doses of AgNPs on BRAF gene expression in four different body organs tissues, after one week and two weeks.

| Group (Dose) | Mean ± SE | | | |
|---|---|---|---|---|
| | Liver | Spleen | Brain | Kidney |
| 0.25 mg/kg for 7 days | 0.0551 ± 0.0232 ab | 0.0078 ± 0.0001 c | 0.0656 ± 0.0278 d | 0.0405 ± 0.0138 cd |
| 0.5 mg/kg for 7 days | 0.0954 ± 0.0276 a | 0.0093 ± 0.0019 bc | 0.2010 ± 0.0458 bcd | 0.1228 ± 0.0309 ab |
| 1 mg/kg for 7 days | 0.0664 ± 0.0270 ab | 0.0293 ± 0.0060 ab | 0.3565 ± 0.0308 bcd | 0.1043 ± 0.0099 bcd |
| 0.25 mg/kg for 14 days | 0.0725 ± 0.0180 ab | 0.0314 ± 0.0122 a | 0.4577 ± 0.1039 bc | 0.1678 ± 0.0216 ab |
| 0.5 mg/kg for 14 days | 0.0507 ± 0.008 ab | 0.0274 ± 0.0111 abc | 0.4815 ± 0.0765 b | 0.1077 ± 0.0061 bc |
| 1 mg/kg for 14 days | 0.0299 ± 0.0072 b | 0.0191 ± 0.0053 abc | 0.9291 ± 0.2464 a | 0.1824 ± 0.0496 a |
| Control | 0.0216 ± 0.0054 b | 0.0169 ± 0.0041 abc | 0.1436 ± 0.0306 cd | 0.0314 ± 0.0037 d |
| LSD value | 0.0538 * | 0.0207 * | 0.317 ** | 0.0736 ** |
| * (P<0.05), ** (P<0.01).<br>In each column, means of different symbols are significantly different. | | | | |

The highest level of liver BRAF gene expression was 0.0954, which achieved after 1 week of daily administration of AgNPs dose (0.5 mg/kg of body weight) (P<0.05), while the lowest liver expression was 0.0299, which came after 2 weeks of daily administration of AgNPs dose (1 mg/kg of body weight) (P<0.05).

The spleen BRAF gene gave 0.0314 as the largest expression level, which came after 2 week of daily administration of only 0.25 mg of AgNPs for each kg of body weight (P<0.05), while the lowest spleen expression was 0.0078 (even lower than spleen BRAF gene expression in the control group), which was seen after 1 week of daily administration of the same dose of AgNPs (P<0.05).

Brain BRAF gene expression reached its maximum value (0.9291) only after daily administration of maximum AgNPs dose (1 mg/kg of body weight) for 14 days (P<0.01), while the minimum value of brain expression of BRAF gene was 0.0656 (even lower than brain BRAF gene expression in control group), which was recorded after daily administration of AgNPs (0.25 mg/kg of body weight) for one week (P<0.01).

The kidney registered 0.1824 as the highest number of BRAF gene expression, and this was achieved after of daily administration of highest dose of AgNPs (1 mg/kg of body weight) for 2 weeks (P<0.01), while the lowest kidney expression was 0.0405, which came after 1 week of daily administration of AgNPs with a dose equal to 0.25 mg/kg of body weight (P<0.01).

## DISCUSSION

As the use of the products containing silver nanoparticles are increased, the possibility of toxicity is propagated on the general health and even on the environment. Recent in vitro and in vivo studies in the vertebrate reveled that AgNPs can harm the immune system, in addition to cellular and genetic toxicity. Moreover, other in vivo and in vitro studies reported that AgNPs can cause variable genetic toxicities even in relatively low or nontoxic doses in several kinds of human or mammalian tissues (43).

The present study showed that there were variable levels of liver BRAF gene expression with the different administered AgNPs dose for the first 7 days, then, there was a gradual decline in the liver BRAF gene expression with the increment of AgNPs dose for the second 7 days. Anyhow, in both durations (7 and 14 days), the overall liver BRAF gene expression level ranges in the 6 different study groups were higher than the expression level in control group.

It is believed that BRAF overexpression can also result in overactivation of the MAPK pathway (44). Hepatic cancer initiation and progression is a complicated process, it includes several steps starting by genetic modifications like oncogenes activation and the suppression of tumor suppressor genes, and proceeds to cause the stimulation of proliferation, differentiation, angiogenesis and other processes which in turn lead to the disruption of essential cellar functions (45). It had been reported that the activation of RAS/MAPK pathway together with the dysregulation of cell proliferation represent the dominant mechanism of hepatocellular carcinoma (46). Other studies suggested that the RAS/MAPK pathway activation is considered the main mechanism of hepatic cancer initiation and progression (47), and about 50-100% of hepatic tumors showed that there was an activation of this pathway (48, 49, 50).

Regarding spleen, the present data showed that there was a gradual increment in the BRAF gene expression

with the increment of administered AgNPs dose for the first 7 days, but then, the next 7 days showed that there was a gradual decline in the BRAF gene expression with the increment of AgNPs dose. In addition, the study groups who received 0.25mg and 0.5mg of AgNPs per kg of body weight for one week showed a spleen BRAF gene expression level even lower than the expression level in brain BRAF gene in control group.

There were two specific mouse models had been generated to study the inducible and endogenous BRAF E600 gene expression (51). The inducible model showed the expression of this gene is associated with increased liver and spleen cellular proliferation, but the other model showed that the gene expression causes an embryonic lethality (52).

The current data reveled that there was a gradual increment in the brain BRAF gene expression with the increment of administered AgNPs dose in the first and second weeks. Furthermore, the study group who received 0.25mg of AgNPs per kg of body weight for one week showed a brain BRAF gene expression level even lower than the expression level in brain BRAF gene in control group.

Some studies approved that BRAF gene alteration can lead to some types of brain tumors, furthermore, they suggested that the growth of these tumors is connected with the MAPK/ERK pathway activation, as that happened in other types of non-brain cancers (53; 54). Raabe *et al*., (2011) established that in addition to its ability stimulation tumor growth, activation of BRAF gene in the brain can also lead to oncogene-induced senescence in some types of brain cancer (55). It had been suggested that there are possible benefits of utilization of BRAF gene targeted therapy in brain tumors treatments (56).

The present study showed that there were variable levels in the kidney's tissues BRAF gene expression with the different administered AgNPs dose for the first and second weeks. Anyhow, as happened with liver BRAF gene expression, the overall expression level ranges of the BRAF gene in the kidney's tissues for both durations (7 and 14 days) were higher in the 6 different study groups in comparison to its expression level in control group.

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the formation and progressive enlargement of fluid-filled cysts, leading to massively enlarged kidneys, interstitial fibrosis and a decline in renal function. In ADPKD, mutations in the PKD genes disrupt intracellular $Ca^{2+}$ homeostasis, relieving BRAF inhibition and allowing cAMP stimulation of the BRAF/MEK/ERK pathway. Archana Raman *et al*., (2018) reported that aberrant BRAF activation is sufficient to induce cyst formation and accelerates disease progression in ADPKD mice (57).

## CONCLUSION

The data generated in this study indicates that the hepatic and spleen tissues expression of BRAF gene is significantly linked to AgNPs administration, also, these data showed a high significant relation between AgNPs administration and the expression of this gene in brain and kidneys tissues. So, these results reflect the possible toxicity of AgNPs which is represented by overactivation of BRAF gene and its harmful consequences in different organs.

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